

## How not to act on good advice

**As European countries engage in diplomatic battle over the banned exports of British beef and its products, lessons can be drawn from the sorry history of the British government's selective responses to expert advice.**

RARELY has the word 'science' passed a politician's lips more frequently than it did last week when John Major, Britain's Prime Minister, told the House of Commons of his plans to retaliate against Europe's refusal to lift a ban on beef by-products. But then, rarely has an issue presented in starker form the morass created when science and politics collide over perceptions of risk. If there are lessons to be learnt from the crisis in the UK beef industry over the bovine spongiform encephalopathy (BSE) epidemic, one is the continued need for responsible ways of handling scientific uncertainty. It is precisely the failure of the British government to do this that has created its current plight.

The act that generated Major's indignation — and has stirred a jingoistic ground-swell in Britain against its European partners — was the refusal of the European Union (EU)'s Standing Veterinary Committee to recommend lifting a ban on the exports of tallow, gelatine and bull semen (see page 353). Formally, the role of the committee, made up of veterinary experts representing the EU's 15 member states, is to provide professional advice on human and animal health. Last week, however, there were few attempts to disguise the extent to which the seven members of the committee who voted against lifting the ban each had broader considerations on their minds than the safety of the product. Britain's chief opponent was Germany, whose demand that British beef be shown to be "completely safe" before any ban is lifted may be unrealistic, but reflects widespread popular concern about 'unnatural' contamination of the food chain.

Whatever the specific reasons, there is plenty to substantiate the criticism of the committee's decision made by Major's beleaguered agriculture minister, Douglas Hogg, that "the language of science has been used to advance political objectives". Nor would many quarrel with Hogg's subsequent suggestion that, "when forming policy, it is extremely important to follow the science". Indeed, the British government has been at pains to emphasize in recent weeks that it has repeatedly followed the suggestions of its scientific advisers.

Speaking literally, the government is correct; most of its advisers' specific conclusions have been acted on. But this argument misses a wider point. Public confidence in today's sophisticated and media-rich world is no longer based on uncritical acceptance of 'scientific' statements — however prominent those who make them — but on a more complex process that hinges on the question of trust. Here, things have gone badly wrong.

The government, for example, accepted immediately the proposal of an earlier advisory committee in the late 1980s, chaired by Sir Richard Southwood, professor of zoology at the University of Oxford, on the need to eliminate beef from BSE-infected cattle from the human food chain. But Germany's stance last week was a reflection less on the British government's intentions than on the effectiveness with which they have been pursued.

There have, in particular, been continued and serious gaps in safety practices in the meat-processing industry. As British ministers recently admitted, spot checks on slaughterhouses carried out as recently as the past nine months have found 25 instances in which pieces of spinal cord had been left attached to carcasses; in at least one case, the entire spinal cord was left attached. During the same period, 12 officials of the Meat Hygiene Service have

been given formal disciplinary warnings for their failure to ensure full compliance with controls on 'specified bovine material'.

Indeed, there is considerable evidence that, if the Southwood committee's broader warnings — and not merely its oversimplified conclusions — had been taken more seriously at the time by both the government and the beef industry, neither would be in the plight it is today. A paper published in *Nature* earlier this month (381, 119; 1996) pointed to the damning fact that almost three-quarters of the 29,000–40,000 cattle likely to develop BSE over the next five years were born after the 1989 ban on feeding animal protein to cattle. One conclusion is that the ban was ignored by some farmers and inadequately policed by the government.

Part of the blame for this must be placed on the political spin given to the Southwood committee's main conclusion — that the chances of humans contracting CJD from BSE were "remote". It took little for John Gummer, the then agriculture minister, to turn this — with the backing of Mrs Margaret Thatcher, the then prime minister — into the assertion that beef was "absolutely safe". One result was to encourage a complacency in the beef industry that played down the severity of the BSE problem. Another was to weaken political support for those demanding a crash programme of research into the possible relationship between BSE and CJD, despite Southwood's secondary conclusion that transmission to humans could not be ruled out, and could prove to be "extremely serious".

The government's announcement last week that an additional £8.5 million is being made available for research by the ministries responsible for agriculture and health implies that this view is (now) shared, albeit in reaction to European pressure. But two tasks still remain. One is a close analysis of the way the warnings of possible long-term health risks — however unlikely — expressed by several respected scientists in the late 1980s appear to have been submerged by a political desire to capitalize on a more simplistic interpretation of its conclusions. This issue strikes at the core of the relationship between government and its scientific advisers, on issues ranging from the deep-sea disposal of the Brent Spar to the safety of radioactive waste. If politicians wish to use the conclusions of scientists to legitimize their decisions, they must be prepared to listen to the full message they are given, not merely isolate those parts that support their own agenda.

As for the beef crisis itself, the British government must now demonstrate the scientific validity of any proposed course of action — in particular, the reasoning behind the strategy it eventually adopts for deciding which cattle should be culled in order to eliminate BSE. It must also gain the confidence of its European partners both in the robustness of its calculations and in its determination to apply them effectively.

Science has little to say on the second of these. On the first, much would be gained if the process were opened up to the scrutiny of an international, politically independent panel, particularly given the continuing uncertainties involved. This could include members nominated by the scientific academies of European and other countries — perhaps modelled on the US National Research Council — and should be guaranteed open access to all relevant medical and veterinary data.



# Britain caught out by 'unscientific' reactions to Europe's beef crisis

**Paris.** Politics triumphed over science again last week in the bovine spongiform encephalopathy (BSE) crisis, when the European Union (EU) flew in the face of its own scientific advice and rejected a partial lifting of an embargo of British beef products. The decision has strengthened calls for a more rational handling of the crisis based on a clear scientific assessment of the risks involved.

But this seems unlikely to happen soon. Much of the European public — and most of the governments — remain deeply sceptical of scientific advice on the matter, perceiving that it has been too slow to acknowledge the possibility that the agent that causes bovine spongiform encephalopathy (BSE) could pass to humans and cause Creutzfeldt-Jakob disease (CJD).

The decision by the EU's Standing Veterinary Committee to reject the lifting of the ban on British gelatine, tallow and bull semen was largely a reflection of the political stance taken by different countries. The committee overruled advice from its own Scientific Veterinary Committee, which said that semen was safe, and that so too were gelatine and tallow, provided that strict controls were used in their production.

The same position was also adopted earlier this month by both the World Health Organization (WHO) and the World Organization for Animal Health (Office International des Epizooties, or OIE), a body that establishes guidelines designed to facilitate trade under safe conditions.

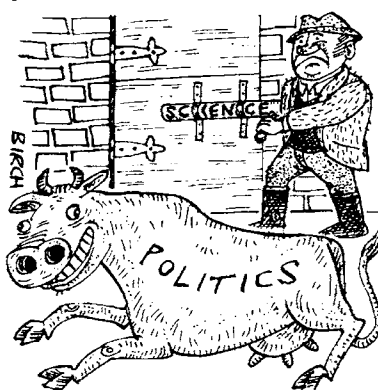
But whereas the EU's Scientific Veterinary Committee is made up of independent experts appointed by the commission, the Standing Veterinary Committee is made up of the chief veterinary officers of their member states, who answer to their political masters.

The commission's proposal to lift the ban failed to obtain the qualified majority it needed to pass, after it was voted against by seven countries — Germany, Austria, Spain, Portugal ('the hard-liners') and Belgium, Luxembourg and the Netherlands. Their reasons for opposing the lifting of the ban were varied, but none can be described as scientific.

Germany and Austria are under pressure from their powerful consumer and ecologist organizations to resist any concessions. Spain and Portugal voted against, not because they were concerned about the safety of beef derivatives as such but in protest at what they claim is insufficient progress in the culling of British cattle. Dutch vets argued that allow-

ing British semen into the country would affect exports of Dutch semen.

The European Commission has since indicated that it will try to bypass the standing veterinary committee's decision and force through a lifting of the ban. In an unprecedented statement, the commission



**BOLTING THE STABLE DOOR AFTER THE MAD COW HAS GONE.**

also said that "the veterinary committee had not respected the scientific data, and had married science and politics too readily".

Meanwhile, Britain has made much of

the unscientific basis for last week's decision, in particular to justify its decision to block other EU business until the ban on derivatives is lifted, and progress is made towards lifting of the wider ban on British beef. But its complaints have received little sympathy in other parts of Europe.

It is pointed out that the British government has only itself to blame for the ban, given that it underestimated the impact of its announcement of a possible link between BSE and CJD, and failed to alert its European partners beforehand. Feeling are running particularly high because, while sales of beef have virtually recovered in Britain, the consumption in the rest of Europe remains badly affected by the crisis.

Indeed, unscientific or not, last week's decision is a gloomy omen of the prospects for scientific advice reasserting itself within the BSE debate, and for the wider ban on beef being lifted. Both the WHO and the OIE say that British beef is safe for consumption provided that certain precautions are taken in its production, even if the hypothesis of a link between BSE and CJD is correct. "Meat is as safe as it could be," says David Hayman director of WHO's ►

## Space agency warned on restructuring

**Munich.** The European Space Agency (ESA) was warned last week that moves to separate planning of space missions from their implementation could jeopardize future space science programmes.

The warning came from David Southwood, professor of physics at Imperial College, London, and head of ESA's Science Programme Committee, in a letter to ESA director-general, Jean-Marie Luton. It follows Luton's presentation of plans for reorganizing ESA to members of the agency's council in Berlin.

Southwood says that he welcomes attempts to improve the operating efficiency of ESA, as required by Europe's space ministers at a meeting in Toulouse last October. He also accepts that separating planning and implementation could increase the efficiency of ESA's 'optional' programmes, such as Earth observation and the international space station.

But Southwood fears that such a move would not benefit the mandatory science programme. This is the only programme to which member states are obliged to contribute, and the resulting long-term predictability of budgets makes reliable

planning possible. Until now, such planning, embodied in the Horizons 2000 programmes, has been carried out by the science directorate, which also controls the programmes themselves. "The director of science [Roger Bonnet] has a complete overview of the programmes, from cradle to grave, and that gives [them] great flexibility and efficiency", says Southwood. Chopping up the directorate horizontally "would throw away the one great advantage of the mandatory programme," he says.

The proposed new management structure for ESA will be discussed by its full council at the end of June. This timing has stirred fears that the reorganization may be carried out with undue haste during the summer.

Ken Pounds, chief executive of the UK Particle Physics and Astronomy Research Council, says that while it would be a "tragedy" if ESA lost the opportunity to streamline its administration, it would be "ridiculous to push through the suggested structure before hearing the considered advice of the scientific community".

**Alison Abbott**



# Jefferson accelerator will seek to pin down quark theory

**Newport News, Virginia.** The world's most powerful electron microscope was inaugurated by Hazel O'Leary, the US energy secretary, last week, enabling physicists to probe the structure of the nucleus and improve their understanding of the nature and function of quarks.

Completion of the \$600 million Continuous Electron Beam Accelerator Facility (CEBAF) on a green-field site in the Naval shipyard town of Newport News marks a considerable triumph for Hermann Gruner, the project director, who took control of the project in 1985. Gruner radically altered its design to incorporate superconducting technology, while at the same time maintaining the political support needed to keep the project on track.

In a robust dedication speech last Friday (24 May), Gruner told his audience of 2,000 staff and invited guests that "times were too easy" when CEBAF got started. "Let me assure you that they're going to get tougher," he said. "We must do a better job of thanking our friends — and obstructing our enemies."

CEBAF, which is managed by a specially created consortium of 41 universities in the southern United States, is already a success in one important sense. The South traditionally fares badly in terms of attracting federal science funds and their associated industrial spin-off. But CEBAF

has already led to the creation of 127 new faculty positions at the 41 participating universities, more than doubling their strength in nuclear physics.

Experiments have been carried out at the facility since last November, when it began firing its continuous beam of electrons at an energy of 4 GeV into stationary targets in one of its three underground target halls. Nearly 550 scientists from more than a hundred institutions will use the beam over the next year, probing nuclei to improve their understanding of how quarks build up the nucleus.

Their experiments will help to verify and refine the theory of quantum chromodynamics (QCD), which defines the 'strong force' holding nuclei together, in a manner analogous to that in which atomic physicists in the 1930s used experimentation to refine quantum electrodynamics (QED).

"Until now, we haven't had a sound scientific basis for understanding the nucleus," says Nathan Isgur, head of theory at CEBAF. Experiments at CEBAF will concentrate at first on the study of the simplest nuclei — deuterium, tritium, helium-3 and helium-4 — as the interactions of protons and neutrons in larger nuclei become alarmingly complicated.

Other electron accelerators, such as that at the Stanford Linear Accelerator Center in California, have delivered short

pulses of electrons, enabling physicists to study a few events at a time. In contrast, the continuous beam at CEBAF will permit the statistical study of hundreds of thousands of collisions every second. A veritable mountain of data will result: in Isgur's analogy, the 300 terabytes produced each year is equivalent to a detailed biography for every human being on Earth.

Roy Holt of the University of Illinois carried out one of the first experiments at CEBAF last November, using the beam to create  $\gamma$  rays with which to split the simplest nucleus — the proton and neutron pair that make up deuterium. Don Geesaman of the Argonne National Laboratory in Illinois set up another experiment, studying the probability that a proton dislodged from a large nucleus will actually pass through it and escape.

During its battle for funding, CEBAF was dubbed the "Warnertron" in honour of Senator John Warner (Republican, Virginia), one of the project's most vigorous patrons, who attended last week's inauguration. Hazel O'Leary, who comes from Newport News, may have been sorely tempted to name it after herself. But she named it the Thomas Jefferson National Accelerator Facility, in memory of the only US president ever to declare that "politics is my duty, but science is my passion".

**Colin Macilwain**

## ►emerging diseases division.

But it still remains difficult to answer critics who argue that the best available scientific advice may not adequately predict future situations, concedes Howard Rees, chairman of the OIE's Code of Commission, and a former UK chief veterinary officer. "We can't say what will happen in ten years time," he admits. "We can only base our decisions on current knowledge; if something new comes along then we have to reconsider."

Germany feels, for example, that the UK government's belated acknowledgement of a possible link between BSE and CJD vindicates its demands since the beginning of the BSE epidemic in the 1980s for stricter precautionary measures to be taken. It is now acknowledged, for example, that the UK's 1989 ban on the use of cattle and sheep offal in feed for these animals was inadequate, in that it stopped short of prohibiting the feeding of offal to any farm animal.

Despite the ban, around 28,000 UK cattle have since become infected, mainly because they ate feed that had been contaminated with pig and poultry feed containing cattle and sheep offal. Only in March this year did the United Kingdom introduce a comprehensive ban on the use of offal in feed for all farm animals. A meeting of the OIE expert group on BSE concluded earlier this month that the new ban

means that now "there should be no risk of infection from feed". Critics ask why such action was not taken sooner.

Suspicion of scientific advice is also being fuelled by the apparent emphasis of British politicians and government scientists in the past on trying to prove the negative case: that BSE could not pass to humans and cause CJD. As a result, much of the public now distrusts messages from scientists, who have been — despite the caveats of some — to have been excessively reassuring.

Indeed, current research into the possible transmission of BSE to humans remains poorly geared to producing answers relevant to public health, according to Annick Alperovitch, head of the French CJD surveillance network and a member of the French and WHO expert committees on BSE/CJD.

Assurances about the safety of eating beef rely too much on experimental data, she says, whereas levels of infection that were so low as to be undetectable in such experiments could still create an epidemic, given the very large numbers of people who may have been exposed.

Similarly, while the WHO and other organizations point out that muscle tissue (beef) has never been shown to be infective, Alperovitch argues that this does not guarantee that infection might not occur under abattoir conditions, for example, through

contamination of beef by other tissues. The difficulty in extrapolating experimental data to the real world explains some of the divergent opinions among experts, she says.

It is important to recognize that science cannot provide certainties within the timescale demanded by politicians and the beef industry, says Alperovitch. "It is important to wait", she says, until epidemiological and other animal experiments clarify the situation towards the end of the year. In the interim period, expert committees will need to steer a course between reasonable precaution and over-reaction.

Whatever the scientific uncertainty about the links between BSE and CJD, many remain convinced that a return to using scientific advice in decision-making provides the only way out of the beef crisis. "My plea is to use more science," says Rees. "It is the only way we can get some rational decisions on this; with politics you don't get rational decisions."

But Rees also agrees that the ban on British beef is unlikely to be lifted soon. Irrespective of what scientific assurances are given as to beef's safety, what many European governments will retain is the hard fact that BSE is still widely present in British herds. They are likely to argue that a reasonable time to lift the ban will be when the UK government has eliminated the disease completely.

**Declan Butler**



# Nuclear stockpile controls under fire in US

**Washington.** The largest programme at the US nuclear weapons laboratories is a "smokescreen" that will not achieve its stated goals of ensuring the safety and reliability of the nuclear weapons stockpile, according to a report published last week by a watchdog group based in Washington DC.

The report, *The Nuclear Safety Smokescreen*, has been produced by the Institute for Energy and Environmental Research (IEER). It uses new data on recorded safety and reliability problems to attack the basis for the Department of Energy (DOE)'s Science-based Stockpile Stewardship programme.

Since the United States stopped testing nuclear weapons in 1992, the \$1.5-billion stewardship programme has become the mainstay of the three nuclear weapons laboratories — Los Alamos and Sandia in New Mexico, and Lawrence Livermore in California. The DOE spends a further \$1.5 billion a year on "stockpile management", which covers the everyday management of the weapons.

The programme concentrates the energies of former weapons designers on establishing how the safety and reliability of the existing weapons will change with age. It includes two major new scientific facilities — the planned National Ignition Facility at Livermore and the Dual Axis Radiographic Hydrodynamic Test Facility (DARHT) under construction at Los Alamos — which IEER says should be cancelled.

The IEER, a technically competent but fiercely anti-nuclear nonprofit group, claims that the DOE has grouped "safety and reliability" together in order to justify the programme. But the technical issues of safety (against accidental detonation) and reliability in service are independent of each other, the report says.

Stockpile stewardship will not be concerned with the safety issues at all, it adds, while the reliability issues it does address will matter only if the United States intends to use its stockpile in a massive 'first strike' against an enemy power.

The DOE defends the stockpile stewardship programme which, it says, will provide the United States with information on safety and reliability which it would once have got through testing, in order to decide how the stockpile should be managed.

"Science-based stockpile stewardship will give scientists and engineers the tools to make these judgements," says Stephen Guidice, a senior DOE official who is now drawing up the environmental impact statement that is intended to provide a public justification for the programme.

Historical data released to the IEER by the department show that none of the 37 safety problems identified in the nuclear primary component of weapons had been attributed to ageing. About a quarter of the reliability problems were attributed to ageing — but 70 per cent of them reduced reliability by one per cent or less.

But Guidice says that the historical information does not provide an accurate guide to the future, as the stockpile was always replenished and only a few years old. Its average age is now 13 years — the oldest it has ever been. "We are now going into an area on which we have very little data," he says. Designers know that the nuclear warheads will change with age as a result of both corrosion and radioactive decay, Guidice says.

James Mercer-Smith, deputy director of nuclear weapons technology at Los Alamos,

adds: "As the stockpile ages far beyond its anticipated life, we can expect a variety of defects which will break the symmetries which were used in the design process".

Arjun Makhijani, president of IEER, says that even if ageing is an issue, the stockpile stewardship programme is not the way to deal with it. Existing monitoring procedures, which withdraw and inspect eleven warheads of each type every year and cut up the nuclear part of one of them to check for defects and remanufacture defective parts as needed, would suffice, he thinks.

Frank von Hippel, professor of Public and International Affairs at Princeton University and a former senior official in the Clinton administration, wrote last year in the *Journal of the Federation of American Scientists* that the programme was a "very costly" concession to the weapons laboratories, offered to secure their support for an end to testing.

But the programme has robust support in the Congress, where many members recognize that its true goal is not the maintenance of the stockpile but the maintenance of the skills of the three weapons laboratories.

**Colin Macilwain**

Telegraph Colour Library



**One less to stockpile:** Trident on test.

## Public faith in science stays high

Washington. The American public continues to hold science in respect, with three-quarters of the population believing that the benefits of research outweigh its harmful results, and little evidence of a strong 'anti-science' mood, according to data gathered by the National Science Foundation.

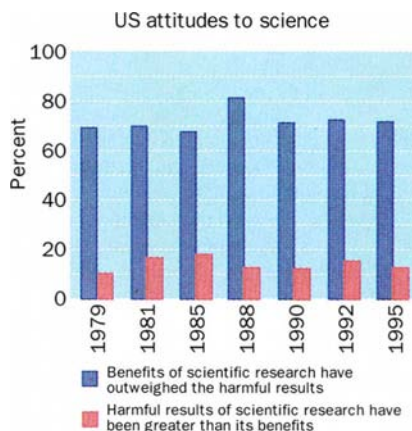
The percentage has held more or less constant since the data were first collected in 1979, and is reported in *Science and Engineering Indicators 1996*, the NSF's massive biennial report on the statistical status of US science, which was published in Washington last week.

Jon Miller, vice-president of the Chicago Academy of Sciences, and main author of the report's section on the public understanding of science, says that there is no trace of the much-touted anti-science movement in the findings. "We couldn't find it before, and we can't find it now — and we've looked very hard for it," he says.

But the public's support for science is not matched by an understanding of how it works, Miller says. Although half the sample, for example, agreed that a clinical trial should put 500 subjects on a drug and keep 500 off it — rather than putting 1,000 people on the drug straight away — almost half of them thought the reason for keeping the 500 on placebo was to save them from the risk of being poisoned.

The report confirms that total spending on research in the United States has been in

steady decline during the 1990s. Industry's investment in research and development (R&D) has held steady at around \$79 billion (in 1987 dollars), but the real value of the government's contribution has fallen 20



per cent since its 1987 peak.

As a percentage of the US economy, this means that US R&D has collapsed from 2.8 per cent of gross domestic product (GDP) in 1991 to 2.4 per cent last year. Much of that decline has been attributable to defence cut-backs, but non-defence R&D has also fallen, from 2.17 per cent in 1991 to 1.94 per cent last year. In an August 1994 policy document, the Clinton administration said that its long-term goal was to raise this last figure to 3 per cent of GDP.

**C. M.**

# French report seeks to slow fusion project

**Paris.** A decision to build the International Thermonuclear Experimental Reactor (ITER), intended to demonstrate the feasibility of nuclear fusion as a source of energy, should not be taken until all the technological problems facing the project have been resolved experimentally, according to an advisory panel to the French government.

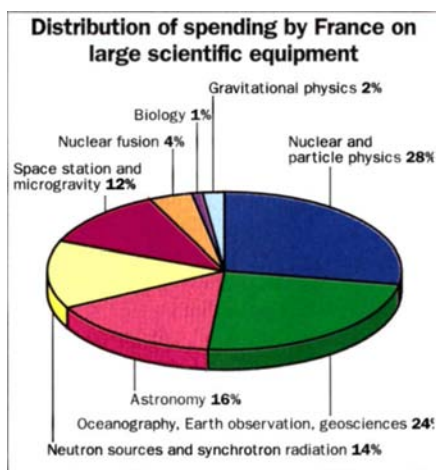
In a report published in Paris last week, it also says the decision should not happen until ITER's long-term financing is guaranteed. At the same it warns that heavy expenditure by France on a European space programme that includes a commitment to the international space station could have a damaging impact on other areas of research.

"There is a sentiment that we need to go more slowly on ITER," says Georges Laurens, secretary of the Conseil des Grands Equipements Scientifiques, which advises the government on large scientific projects.

In its report, the panel argues that the decision due to be taken in 1998 on whether to build ITER needs to be considered within a broader review of the goals of fusion research. Even if ITER gets the go-ahead, it should not be allowed to crush other promising avenues of fusion research, such as basic plasma physics, says the report.

The report also calls for scientists unconnected with ITER to be given a greater say in the running of the project. Laurens says that the need for more external input was "strongly emphasized" by the panel.

The panel's recommendations tie in closely with a growing consensus in both the



United States and the European Union (see *Nature* 377, 567; 1995 & 380, 655; 1996) that the ITER project needs to be reassessed, given its estimated \$10 billion costs.

But although Laurens supports a review of ITER, he also claims much of the opposition to ITER is an "unscientific" knee-jerk response, and warns against what he describes as a growing trend among researchers themselves to attack large-scale science projects on the grounds that the money could be better spent elsewhere.

He points out that funding for such projects is usually extra money provided on top of existing research budgets, and that when planned facilities such as the US Superconducting Super Collider are cancelled, the money saved is usually reallocated to areas

other than research.

Indeed, the report argues that 'mega-science' projects are now the driving force in many areas of research. But Laurens warns that the huge costs of such projects are making international collaboration and hard-headed decision-making vital. (France will spend FF2.4 billion (US\$460 million), excluding staff costs, on large science projects this year, around 10 per cent of state spending on research.)

For example, despite loud demands from European researchers for new neutron sources (see *Nature* 379, 284; 1996), the panel recommends against a spending spree on new facilities, arguing that much of the anticipated needs for neutrons could be met relatively cheaply by upgrading existing sources. Similarly, Laurens says that France should "watch" plans for a European Spallation Source, but "not push" for the facility.

While French and British researchers are keen to build independent synchrotron radiation sources (see *Nature* 381, 100; 1996), the panel swims against the current, describing one machine built by several countries as a "very desirable" alternative.

But in financial terms the report's biggest concern is the impact that the space station will have on France's budget for large-scale scientific projects. Its share of this budget is scheduled to grow from 7 per cent last year to 16 per cent in 1999. If this increase is paid for by making reductions in other astronomy programmes, the effect could be disastrous, says Laurens.

**Declan Butler**

## Freshwater research struggles to keep head above water

**Ottawa.** Canada's federal government has denied claims that funding cuts are likely to lead to the demise of a freshwater research programme described as the "crown jewel" of Canadian environmental science.

Despite rumours to the contrary, L. Scott Parsons, assistant deputy minister for science in Fisheries and Oceans Canada says that the government has no intention of closing either the Freshwater Institute, its Experimental Lakes Area (ELA) programme, or the Canada Centre for Inland Waters at Burlington, Ontario.

Parsons acknowledges that the ELA programme faces a 55 per cent cut in its operating budget and the loss of 21 of its 49 scientific staff over the next five years. But, he says, this forms part of a broader picture of cuts to science programmes.

The overall budget of the fisheries and oceans department is being cut by some 40 per cent, and the science portion of that, amounting to C\$199 million (US\$146 million), is being reduced by C\$71 million.

The budget cuts have also affected other research activities in the fisheries and oceans

department. For example, a 60 per cent reduction has been made in the Atlantic salmon enhancement programme in Canada's maritime provinces, a centre of parasitology in Quebec has been closed, and the department's major oceanographic vessel was mothballed.

The ELA is a collection of 48 small lakes near the Ontario-Manitoba border. The institute has gained worldwide scientific acclaim for its use of the lakes as living laboratories, collecting data over 25 years and studying the effects of pollutants.

The cuts have been strongly criticized from both within and outside the institute. Eville Gorham, Regents' professor of ecology at the University of Minnesota, described the reductions as "a catastrophe". Digby McLaren, a former president of the Royal Society of Canada, said they are "an absolute disgrace".

The reductions led to the resignation last year of Bob Hecky, the scientist in charge of the institute's freshwater science programme. Hecky said that he did not want to preside over what he considered to be the

dismantling of the institute. He added that parallel cuts at the Burlington station would destroy much of Canada's research on the Great Lakes, which hold more than one-fifth of the world's fresh water.

Indeed, Hecky says he believes that the cuts will effectively destroy the institute's entire research programme unless other funding sources are found. He says that although there has been a federal commitment to maintaining the ELA's monitoring programmes, the government has let it be known it will not undertake any new work. But Parsons disagrees and says there was a "moderation" of the cuts last December.

In recent decades, the institute has published pioneering studies of the effects of phosphates and acid rain on aquatic life, and of extremely long-distance airborne transfer of pesticides. A report of ELA work published recently in *Nature* (380, 694; 1996) showed how methylmercury is broken down by light action on surface waters, overturning previous ideas of how mercury makes its way through the environment.

**David Spurgeon**



# Genome ethics panel comes under the microscope at NIH

**San Francisco.** The US National Institutes of Health (NIH) have launched a review of the Ethical, Social and Legal Issues (ELSI) Working Group of the US Human Genome Project, following a change of leadership, disagreement over the control of its budget and a call from at least one working group member for extra funds.

The review will be led by Mark A. Rothstein, professor of law and director of the Health Law and Policy Institute at the University of Houston, Texas, and M. Anne Spence, a geneticist in the department of paediatrics at the University of California, Irvine.

According to Rothstein, the nine-member committee is to concentrate on structural and funding issues, reviewing the working group's relationship with the ELSI programme funded by the NIH and the Department of Energy (DOE). It will also consider its future role in the light of the planned creation by the Clinton administration of the National Bioethics Advisory Commission. Rothstein says that the goal is not to develop a "report card" on the ELSI panel, but to suggest how it might operate most effectively.

The setting up of the review panel follows the recent resignation of the chair of the working group, Lori Andrews, professor of law at the Chicago-Kent College of Law in Chicago. Since then, at least two other members have threatened to resign unless the board is given more autonomy and greater control over its own budget.

But Francis Collins, the director of the National Center for Human Genome Research (NCHGR), points out that the working group's funds come out of the centre's own administrative budget, and that these have already been substantially cut by Congress, to enable more money to be spent directly on research.

The working group is funded jointly by the NCHGR and the DOE. Its goal is to inform the public about ethical, legal and social issues raised by the genome project, and it is funded separately from the ELSI programme, which awards grants for research on ELSI issues. The review committee will look at where financial support for the working group should come from, and how the money should be distributed.

Tensions arose between the working group and the NCHGR earlier this year when the group was told that the centre's budget was sufficient to cover only one meeting this year, compared with three to four a year in earlier years (see *Nature* 380, 96; 1996). Some panel members are also unhappy about disagreements with NCHGR staff over matters such as

whether to comment on the timing of the marketing of genetic tests.

Dorothy Nelkin, professor of sociology and law at New York University, and a member of the working group's executive committee, says that members feel they are sometimes being used to legitimate the genome project, rather than to explore critically issues relating to its social impact.

Collins denies that the working group has been prevented from expressing opinions on controversial topics. But he argues that there has to be "some limits to its autonomy because it is not a free-standing commission". Collins says that the review of its activities will help define the panel's role within the Human Genome Project.

NIH officials say they are particularly upset at claims that moves to commission a set of papers on behavioural genetics had been suppressed because geneticists in the field had been concerned that the panel would put together an attack on their work. "We are not aware of any evidence that such factors played any role whatsoever in the discussions about carrying out this project," says one.

Dan Drell, a biologist in the DOE's Office of Health and Environmental Research who is responsible for the department's liaison with the ELSI programme, said an analysis of behavioural genetics could be an important initiative for the working group, and that it would be of major interest to the general population. But he says that any such analysis would require peer review, and an extremely sensitive and scientifically rigorous approach.

The general review is due to be completed by the end of this year. Drell points out that such external reviews are standard practice for government bodies. "There is a sense that there is a little bit of a lack of focus right now," he said.

Troy Duster, professor of sociology at the University of California, Berkeley, and director of the Institute for the Study of Social Change, is acting director of the working group. He says that, in addition to an explicit mandate, he would like to see the review consider whether ELSI issues overall deserve far more than the three per cent of funding allocated to the Human Genome Project by DOE, and the five per cent set aside by NCHGR.

"The growing gap between diagnostic information and therapeutic capacity is a time-bomb," says Duster. "In this context, the formula for 95 per cent for the mapping and sequencing versus the five per cent for the social consequences seems particularly absurd. What about 50:50?" he suggests.

**Sally Lehrman**

## New Max Planck chief may face a baptism of fire

Munich. Hubert Markl, who takes over as president of the Max Planck Society later this month, may find that his first task is to oversee the closure of entire research institutes or departments in west Germany if Jürgen Rüttgers, the federal research minister, succeeds in imposing planned staff cuts.

In recent years, the Max Planck Society, which runs about 70 research institutes, has been successful in protecting its budget from general cuts in public spending. But since 1994 it has still had to make gradual reductions in its total staff numbers in west Germany in line with the government's policy of reducing all public-sector staffing levels.

As part of Germany's efforts to curb public expenditure in order to meet the "Maastricht criteria" qualifying a country to participate in a single European currency, Rüttgers is now trying to persuade the society to accept further cuts of two per cent a year for the next three years.

But according to its current president, Hans Zacher, the society can cope with this level of staffing cuts only by closing whole departments or institutes. "We have already trimmed all the fat away from our institutes," he says.

The policy of the society has always been to close departments when they are judged to be no longer scientifically productive. In the past, such closures have always been linked to the establishment of departments in new 'hot' scientific disciplines.

But Markl recognizes that he will preside over a period in which the society will no longer have this luxury. Even if Rüttgers' proposal to the Max Planck Society and to the *Länder* governments fails, Markl will still face difficulties.

The five per cent annual increase in the total annual budget, which covers salaries and running and building costs, that has been assured for the next four years, will not cover the full sum required to support the society's expanded activities in the new *Länder* in east Germany as well as continuing all its activities in the west.

Markl is already steeled for a fight, both with the government and, if necessary, Max Planck researchers. He is aware that institutes in the west will resent his decision to continue to protect the building-up of the research infrastructure in east Germany against cuts.

**Alison Abbott**



Markl: may have to close institutes.

# Report stays neutral on deep-sea disposal

**London.** A group of scientists invited by the British government to review the environmental impacts of decommissioning off-shore structures has refused to endorse deep-sea disposal as preferable to other options — a conclusion that is believed to have upset Britain's energy minister.

The panel's report, published last week, also questions the basis used by the Shell oil company to select a site in the mid-Atlantic to dispose of the Brent Spar oil-storage buoy. It recommends that future assessments for decommissioning off-shore structures be carried out by an independent panel of scientists.

But the report also confirms the view put forward by Shell that deep-sea disposal of a structure such as the Brent Spar would have a "very small" overall environmental impact, "roughly equivalent to the impacts associated with the wreckage of a fairly large ship".

The 15-member group was set up at the request of Tim Eggar, a minister in the Department of Trade and Industry (DTI), in the wake of a decision last August by Shell to bow to public pressure and abandon plans for the deep-sea disposal of the 14,500-tonne Brent Spar. Eggar, who made public his disapproval of that decision, remains a strong supporter of deep-sea disposal and is widely thought to have expected the scientists to endorse that view (see *Nature* 381, 99; 1996).

But in the conclusions to its final report, the scientific panel, set up by the Natural Environment Research Council (NERC), and chaired by John Shepherd, director of the Southampton Oceanography Centre, says that nothing in it "should be taken as promoting the deep sea disposal of decommissioned off-shore structures, or of any other wastes".

The report concludes that, from an engineering point of view, "the difficulties and hazards of on-shore disposal are no more than have already been encountered and successfully overcome in other installations". It says that vertical dismantling and disposal on land should not be ruled out.

Despite the relatively low environmental impact of deep-sea disposal, the panel says that "any decision to proceed, or not to proceed", with decommissioning "involves social, economic, aesthetic considerations outside the competence of the group".

Despite having more than six weeks to study the report — publication was delayed by four weeks — the government's response last week was brief. A single-line statement tagged to the end of a press release issued by NERC stated that "the DTI has welcomed the report as a valuable contribution to the decommissioning debate".

A longer response from Eggar was issued later. But no DTI official was available to

answer questions at a press conference held to launch the report — in sharp contrast to the unveiling of a report commissioned by Shell (see *Nature* 377, 670; 1995) which was attended by several DTI representatives. In the latter report, the Norwegian certification agency, Det Norske Veritas, confirmed that the environmental group Greenpeace had overstated the quantity

below the sea. But the report says other sites further afield, which are deeper, softer and 'quieter' — in other words less prone to disturbances such as ocean-bed 'storms' — could also have been considered.

The report also addresses the issue of perceived secrecy surrounding the availability of technical documents used to select the Brent Spar's disposal site. Some documents were belatedly made public; others were classified until recently as "commercial in confidence", according to the report.

EPN/David Sims

For example, when the contract for the site survey was placed, the original agreed requirements between Shell and the government were relaxed, says the report. The original specification required a high-resolution sidescan sonar survey, but this was not carried out. In addition, the results of a low-resolution survey were not made available to the group until February 1996, and omitted a section describing the biology of larger animals.

Eric Faulds, project manager for the Brent Spar decommissioning project, says the UK Scottish Office — "not Shell" — chose the site after considering surveys of three possible disposal sites, one of which was at a depth of more than 3,000 metres.

Commercial confidentiality on some of the documents was maintained, he says, in an attempt to recoup the survey costs. A high-resolution sidescan sonar survey, he adds, could not be carried out because the only vessel capable of undertaking such work was in the Far East at the time.

**Ehsan Masood**

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**Water cannon to the right of them: Greenpeace during last year's battle over the disposal of Brent Spar.**

of waste on board the Brent Spar.

A spokeswoman for the DTI later denied rumours that Eggar was unhappy with the report's findings. The scientists, she said, had been asked to look at the scientific evidence, not to endorse deep-sea disposal. "They are entitled to say what they want. But at the end of the day any decision on decommissioning is for the government to take."

But the report describes as "unclear" the basis on which Shell selected the North Feni Ridge off the west coast of Scotland to dispose of the Brent Spar. The mid-Atlantic site is a hard, pebbly surface 2,300 metres

## When in Rome — watch out for asteroids

**Boston.** With both political and scientific interest growing in potential future collisions between the Earth and either asteroids or planets, a Spaceguard Foundation has been set up in Rome, Italy, to promote the observation of near-Earth objects (NEOs) on potential collision courses.

The nonprofit foundation will coordinate existing NEO searches and sponsor new searches, with the ultimate goal of being an international network of ground-based telescopes similar to that outlined in NASA's 1992 *Spaceguard Survey*.

More specific plans call for the establishment of a coordination centre — where data would be collected, analysed, distributed and archived — possibly at the Minor Planet Center in Cambridge, Massachusetts, now run by Brian Marsden of the Smithsonian Astrophysical Observatory. Longer-term measures include the standardization of hardware and software and the creation of a dedicated

communications network.

The foundation's president, the astronomer Andrea Carusi of IAS-Planetologia in Rome, concedes that the organization has a long way to go before realizing — or even making a significant dent in — its ambitious agenda. At present, the group has access only to a few thousand dollars from membership fees and initial donations. Nevertheless, a fund-raising campaign is being planned in the hope of raising substantial funding from government institutions, space agencies, aerospace companies and private contributions.

The new initiative received a political boost recently when the Council of Europe's Parliamentary Assembly passed a resolution endorsing the establishment of the Spaceguard Foundation, and urging the governments of member states, as well as the European Space Agency, to provide "necessary support". **Steve Nadis**



## UK council backs off plan to 'cap' particle physics bill

**London.** Britain's Particle Physics and Astronomy Research Council (PPARC) has softened its stance on the long-term funding of particle physics. In March, PPARC's council dismayed many physicists when it announced that it was placing a ceiling on overall future spending for the discipline, even though the growing burden of the subscription to the European Laboratory for Particle Physics (CERN) in Geneva could have meant that domestic spending was dramatically reduced.

Last week, however, the council agreed that provision beyond 1997-98 will depend on the outcome both of negotiations with CERN over Britain's subscription, and of a bid to the government for a special injection of £60 million towards the cost of construction of CERN's planned Large Hadron Collider. Meanwhile, domestic spending will be kept level for the next two years. □

## Indian researchers cleared

**New Delhi.** An espionage case against two scientists at the Indian Space Research Organization (see *Nature* 372, 491; 1996) has been dismissed by a court in Kerala following a report by the Central Bureau of Investigation (CBI) clearing both researchers.

After a year-long investigation, the CBI said it had found no evidence that the scientists had passed on designs of India's cryogenic rocket engine to two Maldivian women, as alleged by the state police and the Intelligence Bureau. Nambi Narayanan, one of the accused scientists, said in a statement that "scientists of this country need protection from being treated like third rate criminals based on false allegations". The spying case is believed to have delayed India's cryogenic rocket programme by at least two years. □

## Contraceptive backing urged

**Washington.** Industry and government must jointly commit funds to both basic and applied research if the current "exciting" possibilities for the successful development of new methods of contraception are to be met, according to a report published last week by the Institute of Medicine in Washington.

The report points out that powerful political and social pressures have meant that no products are currently available explicitly designed to prevent or interrupt pregnancy in women exposed to unprotected sex. In the light of the decision by Roussel-Uclaf to abandon its plans to market one such product, RU486, the report says that "it may take partnership between smaller firms and non-profit organizations dedicated to women's reproductive health to bring these controversial products to market, as large pharmaceutical companies may be reluctant to do so". □

## Duma passes science bill

**Moscow.** The State Duma, the Russia parliament's lower house, has adopted a long-awaited bill that provides tax relief for grants and stipends earmarked for science, education and culture. But the bill still needs to be adopted by the Federal Assembly, the upper chamber of the Russian parliament and later signed by the president, before it passes into law. □

## Scripps reaps \$100 million

**San Diego.** The owner of a chain of US drug and grocery stores has donated \$100 million to the Scripps Research Institute in San Diego, California, equalling the record for the largest donation given to medical research in the United States. The donation, from L. Samuel Skaggs of America Store Inc. and his wife, Aline, will go towards establishing the Skaggs Institute for Chemical Biology.

The institute will focus on the study of new approaches to drug

design. The gift equals a \$100-million donation made last year to the University of Utah by Jon M. Huntsman, a chemical industry executive. The new centre will be headed by Julius Rebek Jr, professor of chemistry at the Massachusetts Institute of Technology. □

## Rutherford stays in public hands

**London.** Britain's Daresbury and Rutherford Appleton Laboratories, which provide central facilities for many university-based research groups, are to remain in the public sector as a joint 'non-departmental body' of the Department of Trade and Industry. This decision, announced last week by Ian Lang, the president of the Board of Trade, follows a close examination of various possible future arrangements, including passing the two laboratories, now run jointly as the Central Laboratory of the Research Councils (CLRC), to the private sector.

In announcing his decision, Lang said he had been satisfied that the current arrangements, under which CLRC obtains most of its income from competitive contracts and service-level agreements, "bring suitable market forces to bear on it". He did not, however, rule out "full independence from the public sector" of four institutes of the Biotechnology and Biological Sciences Research Council, including the John Innes Centre and the Silsoe Research Institute. □

## Detectors head for profits

**Paris.** Who said particle physics doesn't pay? First, the European Laboratory for Particle Physics (CERN) gave the world the World Wide Web. Now medical equipment based on a particle detector developed there by Georges Charpak, who won the Nobel prize for physics in 1992, could earn up to US\$80 million a year for Biospace Mesures, a company formed last week to market the devices.

Eurisys Mesures, a leading manufacturer of nuclear equipment, owns 51 per cent of the new company. The remaining 49 per cent share is owned by Biospace Instruments, a company that Charpak founded in 1989 with the l'Ecole Supérieure de Physique et de Chimie Industrielles de la Ville de Paris (ESPCI). □

## Gene panel to be disbanded

**Washington.** Harold Varmus, the director of the US National Institutes of Health (NIH), has announced plans to close down its Recombinant DNA Advisory Committee (RAC), first set up in the late 1970s to provide a regulatory framework for experiments using recombinant DNA techniques.

For the past eight years, the RAC has been reviewing proposed clinical trials of novel gene therapies. But its work has dried up since submission rules were changed last year, and routine approval procedures were passed to the Food and Drug Administration. Recent meetings have been cancelled because of a lack of applications to review. The NIH has asked for public comment on the RAC's closure before implementing its decision. □

## Yeltsin's homage to Sakharov

**Moscow.** The Russian president, Boris Yeltsin, last week laid flowers (right) at the grave of Andrei Sakharov, commemorating the seventy-fifth anniversary of the birth of the dissident physicist and winner of the Nobel peace prize. "Andrei Sakharov was the first teacher of democracy for me and for the whole of Russia," Yeltsin said.

In a meeting at the Kremlin with the physicist and human rights campaigner Yuriy Orlov, Yeltsin pledged to make the defence of human rights a policy priority. In 1976, Orlov announced the formation of the Moscow section of the Helsinki Group for human rights, from Sakharov's Moscow apartment. □

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# Merck, SmithKline and patents

SIR — The position Merck has taken — and publicly demonstrated — on the patenting of genes and gene fragments, as well as access to genomic-based inventions for use as research tools (clones, genes and their expressed proteins, and recombinant cell lines for example) has been broadly misrepresented by Dr George Poste from SmithKline Beecham Pharmaceuticals<sup>1</sup>.

Merck & Co. Inc. recognizes a role for intellectual property protection for genomic inventions in the advancement of biomedical research and the development of new gene-derived therapeutics and diagnostics. But we draw an important distinction between the patentability of genomic inventions and the accessibility of patented genomic research tools to all scientists for research purposes — a distinction not recognized by Poste. Merck believes that patentability and accessibility are mutually compatible. Together, patents and appropriate access maintain incentives for commercial investment in genomic research, while promoting open exchange of scientific information, thereby speeding identification of disease-related genes and development of gene-derived therapies.

Poste misrepresents the Merck position when he states that “Merck claims that in most cases genes and ESTs [expressed sequence tags] are ‘research tools’ that should be placed in the public domain and should not be subject to patenting”, inaccurately citing a publication in *Nature*<sup>2</sup>. This is not Merck’s position, and furthermore confuses patentability with dissemination of basic scientific information.

Merck believes that the requirements of patentability for biotechnology inventions should be the same as for non-biotechnology inventions. Under US patent law, the invention must fall within the definition of patentable subject matter, and must be novel, non-obvious and have utility<sup>3</sup>. As Poste pointed out, patenting of genomic inventions, provided that function and utility are demonstrated, has been widely endorsed, and Merck wholeheartedly agrees. But, in line with patent law, Merck does not believe that patents should be awarded to either genes or ESTs for which the function or utility is speculative or prophetic.

The patent provision of the US Constitution clearly states its purpose, which is “[t]o promote the Progress of Science and the useful Arts, by securing for limited

Times to Authors and Inventors the exclusive Right to their respective Writings and Discoveries...”<sup>4</sup>. Granting patents to genes or gene fragments of unknown or speculative utility would turn this principle on its head, actually denying future inventors the exclusive rights to their discoveries. Thus patents granted for genes or gene fragments based on their unknown future potential would have a destructive impact on the incentives for the industry to research and develop new drugs and therapies based on genomics science. Scientists and financiers, public or private, could invest years of work and hundreds of millions of dollars to identify, say, the gene that expresses a protein that plays an important role in causing Alzheimer’s disease only to find that this particular gene (or fragment thereof) had been locked up years earlier by a speculative patent.

Although Merck seeks patent protection for internally discovered basic research tools, it is a strategy to ensure our continued ability to use those research tools and assays without requiring rights from third parties. What Poste missed — and thus misrepresented — is our allied position on the accessibility of genomic inventions as research tools. Merck’s policy is to negotiate non-exclusive licences for rights under patents to our internally discovered research tools for research purposes by academic scientists at no charge, and to industrial sector entities on reasonable terms as appropriate and consistent with the advancement of biomedical research. Merck’s policy is clear and consistent with the principles of intellectual property protection.

In line with our policy, Merck, in 1994, initiated the Merck Gene Index project to develop a collection of human ESTs, with associated cDNA clones, as a resource publicly available to scientists in both the public and private sectors worldwide. This cooperative effort has already become the largest single contributor of sequence entries — currently more than 75% of the ESTs — to GenBank, the central repository of publicly available gene sequence information at the National Center for Biotechnology Information (NCBI). Curiously, Poste failed to include this contribution among his list of predominant databases of human genetic sequence data, although Craig Venter and his colleagues cited extensive data from the Merck Gene Index project in compiling their initial assessment of human gene diversity and expression patterns<sup>5</sup>. (At 15 April 1996, the Merck initiative had submitted 260,000 ESTs to the public database, from more than 150,000 clones to the public EST database, dbEST.)

The Merck Gene Index project, clearly distinguishing between patentability and accessibility, makes EST data freely available with the aim of promoting the unrestricted exchange of human genomic data, thereby accelerating the rate of discovery in genomics and, we trust, stimulating patentable inventions stemming from subsequent elucidation of the entire sequence, function and utility of the gene.

Interestingly, the National Center for Human Genome Research (NCHGR) recently issued a policy statement in support of this approach. The centre maintains that submitting sequence patent applications may have a chilling effect on research and development, stating that “raw human genomic DNA sequence, in the absence of additional demonstrated biological information, lacks demonstrated specific utility and, therefore, is an inappropriate material for patent filing”.

Public access to sequence data will maximize the probability of discovery of new genes and their function by allowing all who wish to engage in healthy competition to convert that data into new therapeutics. Others, however, have chosen to lock up information about gene sequences and ESTs in the hope that they will have the scientific acumen and luck to translate sequence information into successful biotherapeutics. We may not know for a decade which approach will produce more ‘winners’: it is Merck’s contention that broad and open access means a win for science and discovery, and ultimately, the future of health care.

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## BSE a specific bovine disease?

SIR — It has been postulated that BSE is not a variant of scrapie accidentally acquired by *Bos taurus* but more probably a specific bovine disease associated with cows.

Scrapie has been known for more than 200 years, but a French veterinary surgeon in southern France, M. Sarradet, described as early as 1883 “a case of scrapie in an ox” (*Rev. Vétérinaire* **3**, 310–312; 1883).

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# Complementary medicine

SIR — Complementary and alternative medicines are more popular than ever before. A recent Australian survey suggests that about half of the general population employs such treatments<sup>1</sup>. This is remarkable when we know so little about the efficacy

damage such as spinal cord transection and 1 disk protrusion.

Such survey data are inevitably limited. The first questionnaire was likely to be completed by relatively healthy proponents of CAM and not by individuals with experi-

SUSPECTED ADVERSE EFFECTS OF COMPLEMENTARY/ALTERNATIVE MEDICINE

| Therapy         | Users survey    |                                                                                       | GP survey       |                                                       |                                                          |
|-----------------|-----------------|---------------------------------------------------------------------------------------|-----------------|-------------------------------------------------------|----------------------------------------------------------|
|                 | % Reporting AEs | Most commonly reported AEs                                                            | % Reporting AEs | Most commonly reported minor AEs                      | Most commonly reported major AEs                         |
| Manipulation    | 15.8 (24)       | Pain (13)<br>Fatigue (2)<br>Dizziness (3)                                             | 15.4 (71)       | Aggravation (65)                                      | Fractures (3)<br>Nerve damage (2)<br>Disk protrusion (1) |
| Acupuncture     | 12.5 (23)       | Aggravation (6)<br>Mental effects (3)<br>Fatigue (3)<br>Pain (2)<br>Needle trauma (2) | <0.1 (4)        | Aggravation (2)<br>Visual disturbance (1)             | Septic arthritis (1)                                     |
| Homeopathy      | 9.8 (28)        | Aggravation (11)<br>Mental effects (3)<br>Digestive effects (2)                       | <0.1 (9)        | Aggravation (5)<br>Eye infection (1)<br>Skin rash (1) | Liver failure (1)<br>Anaphylaxis (1)                     |
| Herbal medicine | 7.6 (10)        | Digestive effects (3)                                                                 | <0.1 (6)        | Aggravation (2)<br>Rashes (2)<br>Nausea (1)           | Liver toxicity (1)                                       |

Numbers in parentheses show absolute number of cases.

cy and safety of these therapies. It is commonly believed that complementary/alternative medicine (CAM) is natural and therefore safe and that people can be treated without side-effects. The results of two recent surveys, however, provide evidence on the potential risks.

In the first study, we invited readers of the British daily newspaper *The Guardian* (circulation about 500,000) to answer a questionnaire about their experiences with CAM. The 386 respondents were, as expected, strongly in favour of its use: 91.4% had a positive attitude, and 95.6% said it had improved their quality of life. But when asked about treatment outcome, a large proportion (23.8%) reported adverse effects (AE) (see table). The rate of AEs following acupuncture (12.5%) was similar to that reported elsewhere<sup>2</sup>.

In the second study, a questionnaire was sent to every general practice in Devon and Cornwall ( $n = 972$ ) enquiring whether general practitioners (GPs) had encountered patients experiencing problems with complementary therapies. Of the respondents, 176 (38.2%) reported encountering AEs. The table describes the direct physical AEs, but there were also 11 reports of psychological effects (mainly disillusion at absence of promised benefit) and 17 cases of inappropriate management or frank misdiagnosis by complementary therapists. Manipulation therapy, such as chiropractic and osteopathy, was the dominant cause of physical AEs seen by GPs (see table): 3 of these cases (1.3% of all AEs reported) led to bone fractures and 2 to neurological

ence of serious AEs. The GP survey, on the other hand, was likely to collect reports of the more severe AEs. Furthermore, neither questionnaire provides reliable prevalence figures. However, both sets of data illustrate that CAM is not entirely free of risk. We therefore suggest more rigorous investigations into this topic to determine the extent of the problem and (if necessary) to design a strategy to minimize harm.

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## Feynman faux pas

SIR — Our fascination with Richard Feynman's lectures (see review of *Feynman's Lost Lecture*<sup>1</sup>) is fuelled in part by the glimpses they give us of his extraordinary mind. Readers of *Nature* might therefore relish learning that Feynman could blunder.

In a lecture at the US National Academy of Sciences in 1955 (reprinted in ref. 2), Feynman muses about why writers and artists do not rhapsodize about science. He wonders if perhaps they don't know "how to read the music", which he suggests is necessary to develop beautiful abstract ideas. He

then invents an example in which ironically he, of all people, appears to misread the music. Feynman says (page 244): "For instance, the scientific article may say, 'The radioactive phosphorus content of the cerebrum of the rat decreases to one-half in a period of two weeks.' Now what does that mean? It means that phosphorus that is in the brain of a rat — and also in mine and yours — is not the same phosphorus as it was two weeks ago."

He tries to make something of the fact that our mind retains information despite replacement of phosphorus atoms in the brain. But the example he gives would not prove replacement; in fact, it suggests quite the opposite. In an endearing lapse, Feynman appears to have blanked out on the half-life for the decay of <sup>32</sup>P to sulphur, which just happens to be two weeks; if the radioactivity decreases to one-half in two weeks it means (within experimental error) that there was no turnover of phosphorus in the cerebrum.

A further irony is that the excerpt is cited approvingly by Daniel C. Dennett in his much-praised book on evolution<sup>3</sup>; Dennett is also concerned about scientific abstraction and introduces the quotation with reckless adulation, saying (page 360), "Nobody has ever put it better than the physicist Richard Feynman".

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## Hard evidence

SIR — It has been suggested that the problem of consciousness consists of an 'easy problem' and a 'hard problem'. Exploring the neurobiology of vision, for example, is 'easy' but understanding subjective visual experience is 'hard'<sup>1–3</sup>. To solve the hard problem, a 'new theory' has been developed in which 'conscious experience' is an irreducible phenomenon, with a physical (brain state) and an experiential (subjective state) aspect linked by psychophysical laws<sup>3</sup>. Replace 'conscious experience' with 'unbewusstes Schluss' and this is the theory presented by von Helmholtz in 1857 (ref. 4). Progress in the study of consciousness has not been delayed by theoretical constraints.

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# Biotechnology and Brown Shirts

SIR — Scientists should find it chilling that “[l]ast year, all 15 small-scale field trials conducted by universities and research institutes in Germany were partially or fully destroyed by activists, even though most of those experiments were studying the environmental safety of growing genetically manipulated plants in normal agricultural environments” (*Nature* 380, 94; 1996).

It recalls Germany in the 1930s, when the Third Reich vilified and persecuted the practitioners of what the regime called *Entartete Kunst*, “degenerate art”. These subversives included Marc Chagall, Emil Nolde, Max Beckmann, Henri Matisse, Edvard Munch and Pablo Picasso.

Now we have a kind of *Entartete Forschung*, “degenerate research”. The German government is not culpable, except indirectly by neglecting to protect the personal safety and property of plant scientists against assaults by anti-biotechnology activists.

But the vandals are abetted by government ambivalence and policies that equate innovation with risk. There is an obvious solution — one that has been purposefully ignored by policy-makers in both the European Union (EU) and Germany: simply apply scientific and risk-based regulatory policies to the testing of transgenic plants.

As *Nature* said in a leading article in 1992 (356, 1–2), a broad scientific consensus holds that “the same physical and biological laws govern the response of organisms modified by modern molecular and cellular methods and those produced by classical methods.... [Therefore] no conceptual distinction exists between genetic modification of plants and microorganisms by classical methods or by molecular techniques that modify DNA and transfer genes.”

Disregarding the scientific consensus, current EU and German regulation requires extensive case-by-case government environmental assessments for proposed field testing with recombinant-DNA-manipulated plants. By contrast, phenotypically similar or identical plants are usually not subject to government scrutiny or requirements (or publicity) at all. And that applies even to the many new plant varieties that result from ‘wide crosses’, hybridizations in which genes have been moved from one species or genus to another — ‘transgenic’ plants by any reasonable scientific definition.

If recombinant-DNA-manipulated plants were treated like other new varieties, their testing would not need government review, special warning signs or public announcements. There would be no way for the thugs

to target and disrupt field research that they have decided are *Entartete Forschung*.

There is an important lesson here: the problem would have been avoided entirely if public policy had been crafted intelligently in the first place.

**Henry I. Miller**

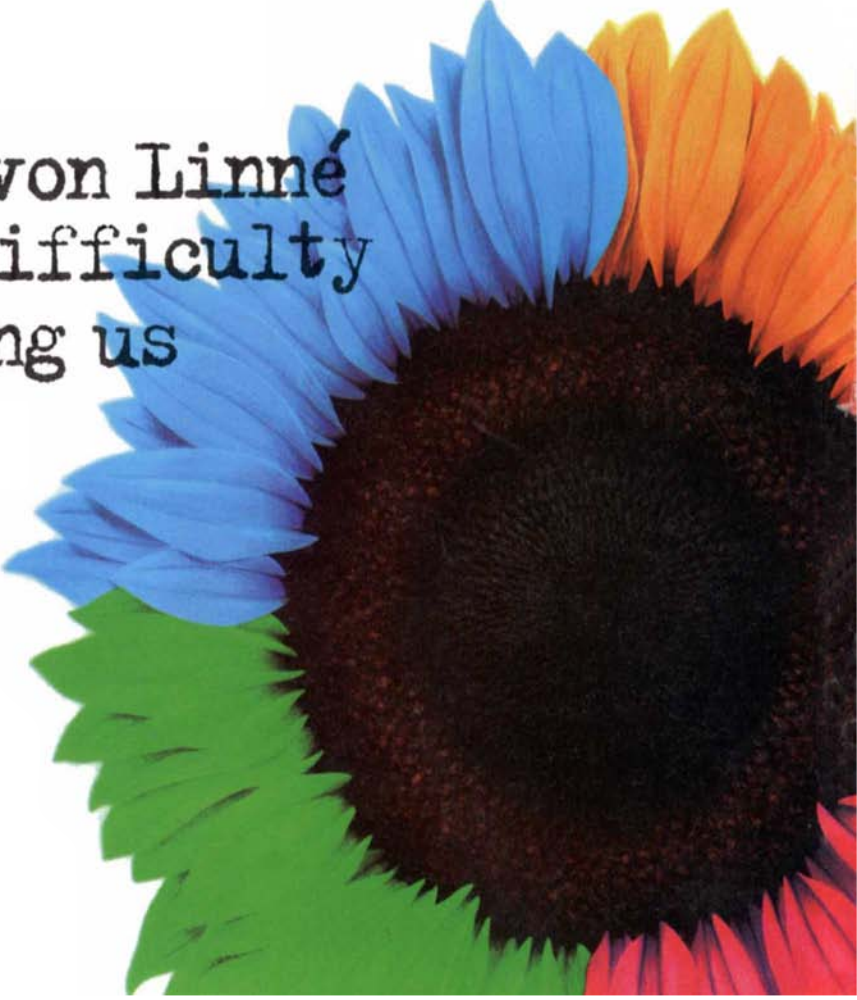
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## Mild conversion?

SIR — The gentler tone of John Maddox (*Nature* 378, 435–437; 1995) would almost seem to represent a mild conversion. His appeal to religious scientists to help his unloved colleagues is quite touching.

If there really is serious distrust of science in our society, there are two matters on which we might meditate a little. The first is rather prosaic, but causes persistent annoyance. Listening to Wolpert or Dawkins, for instance, one is aware that some of the senior practitioners consider themselves, as scientists, to be in a very special category of human being. Rationality is their special preserve; and those outside science are assumed to be incapable of following their levels of logic. To me, this is both sad and nonsensical. By and large, I would say that scientists deal with the easier problems. In



Even Carl von Linné  
would have difficulty  
classifying us



fact, they deliberately select those problems that may lead to some specific solution. Others — historians, teachers, nurses, lawyers, architects, musicians, or whatever — work to equally demanding disciplines, and do not have the luxury of selecting easier options. And they are generally sensible enough to see their work as part of the overall culture. I see no need for any special status for scientific activity.

The more serious point I would make is that this century will be seen by future historians, not as Maddox would like us to view it, but as quite the most barbaric in all history. The two world wars, in which all sides indulged in indiscriminate slaughter, had their origins to some extent in scientific notions — 'survival of the fittest' and genetic purity. Also, there was never the slightest difficulty in recruiting leading scientists to design lethal weapons. Given money and security, scientists, like most other people, will do what is asked of them. There are very few exceptions.

With the knowledge and power we now possess, we need so much more than the abstract, impersonal logic of science. Those who wish to make an alternative religion of Darwinism and reductionism, and encourage belief in the pointlessness of existence, seem to have learnt nothing from the history of this century.

Maddox's final quixotic comment — there are still molecular biologists who go to

church — reveals much. To those of us not wedded to Darwinian dogma, one might just as well say that there are scientists who still read Plato. We may have cells and DNA that have much in common with the animal world. But in our mentality — which has apparently arisen spontaneously in the last few moments of evolutionary time — we are light years ahead of any other organism. Externally, we have transformed the Earth, creating cities and civilizations; internally, we compose symphonies and formulate predictive mathematical theories. At our best, we think seriously about purpose and meaning, and try to assess the human and social consequences of our work. In this, science provides no guidance, and we each have to find our own path. Religious thought provides one option.

**John Evans**

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## Smoke them out

SIR — Precious research funds are being spent on attempts to identify means of deterring people from smoking. I have a suggestion that might yield economies. In Britain at least, young people, especially women, continue to smoke despite warnings by public health authorities of the dangers. The fault may lie in the messages that adorn

cigarette packages and advertising hoardings. "Harm your baby" or "Cause fatal diseases" are anodyne if not challenging to young people. They see smoking as fashionable, romantic, anarchic and exciting: all the supermodels smoke, as do Hugh Grant and Elizabeth Hurley, futures traders and Porsche drivers<sup>1</sup>.

My suggestion is this. Smoking is associated with urinary incontinence in women<sup>2,3</sup>. Moreover, one may assume that the association is causal because transdermal oestrogen is effective in the treatment of mild to moderate stress urinary incontinence in postmenopausal women<sup>4</sup>, and cigarette smoking is anti-oestrogenic<sup>5</sup>.

Cigarette packets should therefore carry the message "Cigarettes cause urinary incontinence". Young people may be half in love with easeful death, but easeful incontinence is a different matter. Once smoking becomes associated with incontinence pads, it will become less fashionable.

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professor, lecturer and a resident of the Swedish university city of Uppsala (pronounced OOP-SA-LA). A consummate classifier, Linné systematized the plant, animal and mineral kingdoms as well as drew up a treatise on the diseases known in his day.

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# A new strategy for genome sequencing

*J. Craig Venter, Hamilton O. Smith and Leroy Hood*

**Existing approaches to sequencing the human genome are based on the assumption that each region to be sequenced must first be mapped. But there is a simpler strategy in which any number of laboratories can cooperate.**

ONE of the main goals of the Human Genome Project is to sequence, in an international cooperative venture, the 3 billion or so base pairs of DNA that make up the 24 different human chromosomes. The order of the bases along each chromosome (the sequence maps) will allow the identification of the 80,000 or so human genes and provide a framework for studying how certain DNA variations among humans predispose towards various diseases. The project was initiated in 1990 by the US government (the National Institutes of Health and the Department of Energy) and has been joined by the United Kingdom, France, Germany and Japan. Its estimated cost is \$3 billion over 15 years.

The first five years have focused on genetic and physical mapping<sup>1</sup>. The genetic map is a representation of the order of the genes along the chromosomes; the physical map is a collection of identifiable overlapping fragments of DNA, together with a specification of how they are arranged along the chromosomes.

We are now entering the more complex sequencing phase<sup>2,3</sup>. Existing approaches to sequencing the human genome are based on the assumption that each region to be sequenced must first be mapped. Here we argue that this step is dispensable. The alternative approach we propose makes it possible for any number of laboratories to cooperate in the effort, aiding international collaboration between large genome centres and small groups, and should be simpler and cheaper than map-based strategies. What is more, it will help the sequencing of biologically interesting chromosomal regions such as gene families (encoding, for example, neural and olfactory receptors), as well as smaller genomes of simpler organisms.

The most common approach to sequencing the human genome uses a three-stage divide-and-conquer strategy (see the figure). It involves the construction of three different clone libraries from human chromosomal DNA. This is done by randomly cutting the DNA into fragments, separating these into differing size classes and then inserting the fragments into distinct vectors capable of

propagating them in appropriate hosts such as bacteria or yeast (see the table). (A clone here comprises a vector with a single inserted fragment of human DNA, whereas a clone library comprises the

pairs per clone) and assembled computationally into the sequence of the 40-kilobase (kb) cosmid insert. This random, or 'shotgun', approach ensures a high degree of accuracy because every nucleotide is sequenced about eight times (400 bases per clone  $\times$  800 clones = 320,000 bases of sequence). Most genome-wide or chromosome-specific physical maps generated so far are of low resolution and based on YACs<sup>4,5</sup>.

Hank Morgan/SPL

The approach to genome-wide sequencing presents several challenges and has various limitations. First, initial attempts at high-resolution mapping of human chromosomes 16, 19 and 22 have been expensive and are still not finished; the problem is that it is difficult to obtain complete maps without any gaps. Completing sequence-ready maps for these and the other human chromosomes remains a formidable task.

Second, some 50 per cent of YAC clones show structural instability of inserts, resulting in deletion or rearrangement of portions of the cloned DNA, or are chimaeras in which two or more DNA fragments have become incorporated into one clone. These defective YACs are invariably unsuitable for use as mapping and sequencing reagents, and a great deal of effort is required to identify them. Cosmid inserts sometimes contain the same aberrations and are similarly difficult to detect.

Third, the human genome contains tandem (adjacent) arrays of DNA units with high sequence similarity (for example, five tandem 21-kb arrays) and tandemly arrayed genome-wide repeats (for example, the 7-kb long interspersed nuclear elements) that pose problems for high-resolution mapping when the size of the clone insert is less than that of the tandem array because the landmarks are similar, such as a 40-kb cosmid insert against a 105-kb DNA array.

Fourth, the conventional sequencing procedure is complex and difficult to automate fully for the vast amount of sequencing we hope to achieve in the future.

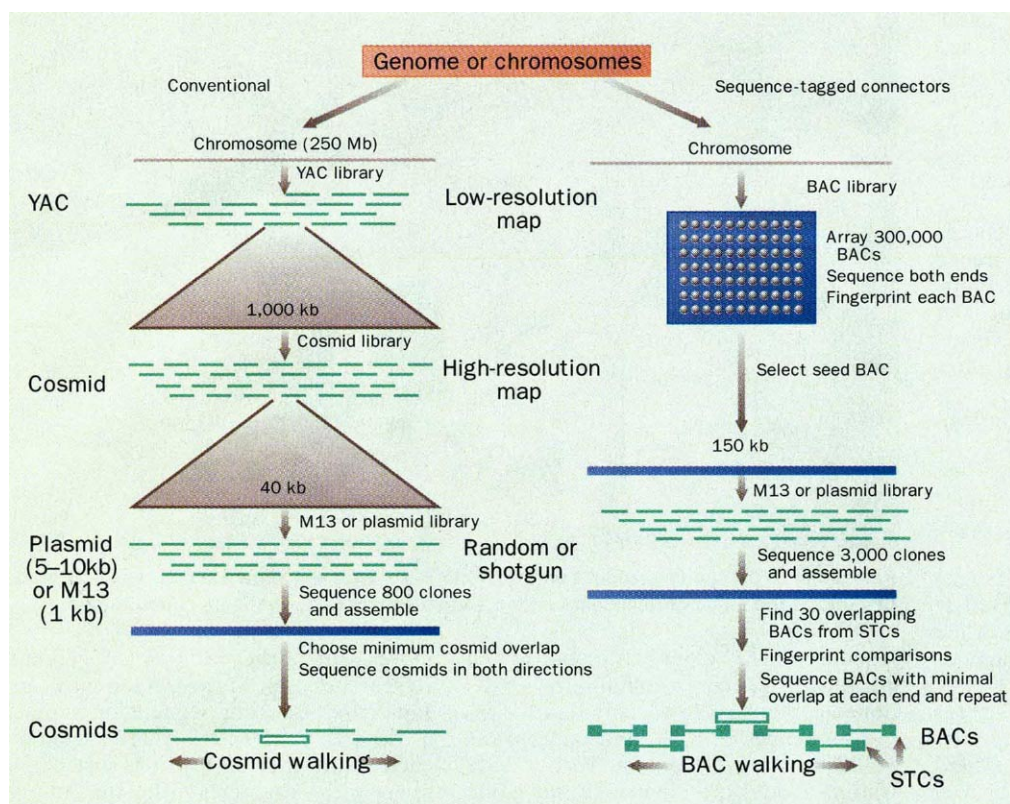
Finally, because an extensive infrastructure is required for high-resolution physical mapping, collaboration among

## The Human Genome Project is now entering the more complex sequencing stage.

entire collection of human DNA fragments each inserted into a vector molecule.)

Low-resolution physical maps of each chromosome are first prepared by identifying shared landmarks (such as unique sites that can be amplified by PCR (sequence-tagged sites or STSs) or restriction-enzyme digestion sites) on overlapping yeast artificial chromosome (YAC) clones. High-resolution or sequence-ready maps are then made by randomly cutting and subcloning YAC inserts into cosmid vectors, and a map is constructed by identifying their landmark overlaps. Next, a minimally overlapping path of cosmid clones is chosen and the DNA from each clone is randomly fragmented into small pieces and subcloned into M13 phage or plasmid vectors. For each cosmid, about 800 M13 phage clones are sequenced (roughly 400 base





The conventional sequencing approach and the newly proposed sequence-tagged connectors (STC) approach. The bacterial artificial chromosome (BAC) clones in the STC approach could be sequenced by any cost-effective strategy.

large and small groups is difficult.

These problems, and two important scientific advances, lead us to propose a new strategy for cooperative sequencing of the human genome. The first advance is that we can now sequence and assemble prokaryote genomes of up to several megabases (Mb) with high accuracy and fidelity<sup>6,7</sup>. The second is the development of bacterial artificial chromosome (BAC) libraries that can accept human inserts of up to 350 kb. BAC clones seem to represent human DNA far more faithfully than their YAC or cosmid counterparts<sup>8,9</sup>. For example, the 1-Mb locus encoding the human  $\alpha\delta$  T-cell receptor has been mapped using only 17 BAC clones, in contrast to the 75 or so cosmid clones that would have been required for the same coverage. Detailed landmark analyses show that only one of 17 BAC clones had a defect, a small 6-kb deletion (C. Boysen, personal communication).

What is more, BAC clones are excellent substrates for shotgun sequence analysis. For example, five out of five BAC clones, ranging in size from 89 to 210 kb, have been sequenced using this approach (C. Boysen, personal communication); other laboratories have had similar success rates. So BAC clones seem ideal for producing an accurate contiguous sequence.

Our new approach to genomic sequencing eliminates the need for any prior physical mapping and uses BAC

clones as the basic sequencing reagent (see the figure). A human BAC library with an average insert size of 150 kb and about 15-fold coverage of the human genome contains 300,000 clones. These are arrayed into microtitre wells. Both ends (starting at the vector-insert points) of each BAC clone are then sequenced to generate 500 bases from each end. The 600,000 BAC end sequences are scattered roughly every 5 kb across the genome and make up 10 per cent of the genome sequence. We denote them 'sequence-tagged connectors', or STCs, because they allow any one BAC clone to be connected to about 30 others (for example, a 150-kb insert 'divided' by 5 kb will be represented in 30 BACs). The

reactions).

■ The BAC clones can be made readily available to sequencing groups worldwide through resource centres and commercial distributors. Large centres could sequence many different BAC clones forming major contiguous regions of DNA, while small groups could contribute one or a few BAC sequences.

■ As improved techniques for generating BAC or other yet-to-be-developed libraries appear, reasonable numbers of these new clones could easily be added to the clone collection.

■ It is likely that our approach will eliminate the problem of generating complete maps without gaps for high-resolution physical mapping.

CLONE LIBRARIES USED FOR GENOME MAPPING AND SEQUENCING

| Vector                                | Human-DNA insert size range | Number of clones required to cover the human genome |
|---------------------------------------|-----------------------------|-----------------------------------------------------|
| Yeast artificial chromosome (YAC)     | 100–2,000 kb                | 3,000 (1,000 kb)                                    |
| Bacterial artificial chromosome (BAC) | 80–350 kb                   | 20,000 (150 kb)                                     |
| Cosmid                                | 30–45 kb                    | 75,000 (40 kb)                                      |
| Plasmid                               | 3–10 kb                     | 600,000 (5 kb)                                      |
| M13 phage                             | 1 kb                        | 3,000,000 (1 kb)                                    |

■ The existing chromosomal landmarks, STSs (PCR-specific sites) and expressed sequence tags or ESTs (partial complementary DNA sequences), can be easily placed on the BAC clones, adding additional markers for BAC clones and taking advantage of any associated biological information.

■ The 10 per cent of the genome obtained in the STCs can be searched against the sequence database to identify many interesting landmarks (such as genes, STSs and ESTs) that could locate the BAC clone on the preexisting chromosomal maps.

■ Chromosomal regions of key biological interest can be sequenced first.

■ The human genome can be sequenced earlier and for less money (with savings on, for example, high-resolution physical mapping).

■ The STC approach will provide useful clones for biological studies even at the early STC sequencing stages when only three- to fourfold coverage is achieved.

■ This would be an efficient strategy for sequencing genomes of smaller organisms such as single-cell eukaryotes and the malarial and other parasites, as well as the model organisms that the genome project is already committed to sequencing: the bacterium *Escherichia coli*, the nematode, the flowering plant *Arabidopsis*, the fruitfly *Drosophila* and the mouse. (The yeast genome is finished.)

Arrayed and DNA preparation facilities could readily make the end-

**Release of STCs and fingerprints on the World Wide Web will allow several research teams to work on the same chromosome region and so enhance international cooperation.**

sequenced BAC clones available to the worldwide genome community. BAC clones could be easily mailed and BAC end sequences or STCs and fingerprints would be available on the World Wide Web, as would the identity of any clone selected for sequencing. Several research teams could therefore work on the same chromosomal region without unintended duplication of effort. This would aid international cooperation. With our proposed strategy, participating laboratories could sequence the BAC inserts by any cost-effective method they choose. Similarly, any DNA sequencing chemistries could be used, including those not yet developed.

Peter Menzel/SPL

The complete set of BAC end sequences and fingerprints could be obtained in two years or less using, for example, 30 Applied Biosystems 377 sequencers at a total cost of \$5–10 million. This is a small fraction of the cost of sequence-ready physical mapping that has yet to be incurred.

A highly cooperative combination of large genome centres and small groups could finish sequencing the entire human genome in under ten years. The current cost of DNA sequencing is around \$0.30 per finished base pair in the most efficient laboratories, and it is expected that it will fall to \$0.10–0.25 per base pair in the next one to three years. At these rates, the total sequencing cost for the entire genome would be less than the genome funds spent so far. The Wellcome Trust in the United Kingdom recently announced that it was funding the Sanger Centre to sequence a sixth or

more of the human genome. Researchers in France, Germany and Japan are discussing sequencing as much as 10 per cent of the human genome each, while the US effort is just beginning with the establishment of six genome sequencing centres for pilot scale-up studies<sup>11–14</sup>. These large centres around the world, together with many smaller groups, require an improved strategy for genome coordination. The STC strategy proposed here offers a powerful new approach to sequencing human and other genomes, with maximum international cooperation and with all participants working on an equal basis in a self-regulating, open scientific effort. □

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**With the STC strategy, participating laboratories could sequence the BAC inserts using any DNA sequencing chemistries, including those not yet developed.**

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# How to tell a cell where it is

Jim Smith

**In the embryo, cells have to be aware of where they are so that they can differentiate into the right tissue. In part, long-range signalling is involved, but it is not yet clear how diffusible morphogens specify cell type.**

MENTION 'positional information' to most developmental biologists, and you could be in for a long conversation. In one view of events, outlined almost 30 years ago<sup>1</sup>, cells discover where they are in the embryo, and differentiate accordingly, by referring to the concentration of a diffusible 'morphogen'. The morphogen is thought to be secreted from a localized source. If a cell is close to that source, it will experience a high concentration of morphogen and activate one set of genes; if it is further away, it will experience a lower concentration and activate a different set of genes.

The appeal of this idea lies in its simplicity and in its ability to explain diverse experimental results<sup>2,3</sup>, but even after three decades definitive proof remains elusive. Of course, this may be because diffusible morphogens do not exist. But in truth it is difficult to distinguish experimentally between a diffusible-morphogen model and a 'relay' system, in which the long-range effects of a localized signal are achieved through sequential interactions between adjacent cells.

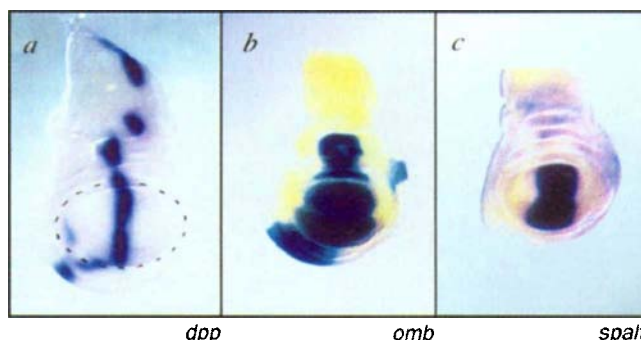
Some of the best evidence that diffusible morphogens do indeed exist comes from studies on the frog, *Xenopus*, where the mesoderm-inducing factor activin appears to diffuse from a localized source to activate expression of the genes *goosecoid* and *Xbra*<sup>4,5</sup> (see also refs 6, 7). The effects of activin in this assay can traverse a barrier of cycloheximide-treated cells, making it unlikely that a relay mechanism is in operation. But it nevertheless remains possible that these cells transmit a positional signal despite being unable to synthesize protein.

Two groups studying wing development in *Drosophila* have now resolved much of this controversy. In papers published in *Cell* and on page 387 of this issue, Nellen *et al.*<sup>8</sup> and Lecuit *et al.*<sup>9</sup> show that a signalling molecule can indeed act at a distance, presumably by long-range diffusion. There remains some disagreement, however, in deciding exactly how this molecule activates expression of the right gene in the right place.

In the developing *Drosophila* wing, the product of the *decapentaplegic* (*dpp*) gene

is expressed (in response to the short-range signalling molecule hedgehog) in a line of cells separating the anterior and posterior compartments of the wing imaginal disc — the part of the larva that gives rise to the wing in the adult. From this position, *dpp* exerts a long-range organizing effect in both compartments. If, for example, *dpp* is overexpressed in its normal expression domain, both halves of the wing become broader.

These effects could occur through long-



Normal expression patterns of three genes active in the imaginal disc from which the *Drosophila* wing develops. *a*, *Decapentaplegic*; *b*, *optomotor-blind*; *c*, *spalt*. Note that the expression domain of *optomotor-blind* is wider than that of *spalt*. (Reproduced from ref. 8.)

range diffusion of Dpp protein or by initiation of a relay mechanism. To investigate this issue, it was first necessary to identify potential target genes of Dpp protein, as Gurdon and his colleagues did in their analysis of activin signalling in *Xenopus*<sup>5</sup>. In *Drosophila*, two such targets prove to be *optomotor-blind* (*omb*) and *spalt*<sup>10,11</sup>. Neither gene is expressed in wings which lack functional Dpp, and both are required downstream of Dpp during wing development. In the normal wing imaginal disc, *omb* and *spalt* are both expressed in broad stripes centred on the *dpp* expression domain, but the *omb* domain is more extensive than that of *spalt* (see figure).

From these observations, the classic diffusible morphogen model would have it that gradients of Dpp protein are set up in the anterior and posterior halves of the wing. Expression of *omb* would be activated at low Dpp concentrations and expression of both *omb* and *spalt* would occur at high concentrations.

Earlier this month, Nellen *et al.*<sup>8</sup> provided evidence that this is precisely what happens. If marked clones of *dpp*-expressing cells are created in the wing disc,

haloes of *omb* and *spalt* expression are induced in surrounding non-*dpp*-expressing cells, with the *omb* halo being more extensive than that of *spalt*. This is most simply explained by Dpp diffusing from its site of synthesis into surrounding cells and activating both *omb* and *spalt* at high concentrations and just *omb* at low concentrations. Two other experiments are consistent with this idea. First, if *dpp* is expressed ectopically (that is, in the wrong place), under the control of a weak promoter, it is only able to activate expression of *omb* in neighbouring cells, not *omb* and *spalt*. Second, if *dpp* is overexpressed in its normal expression domain, the extent of *spalt* expression is significantly extended.

Although compelling, these experiments still do not rule out a relay model. So Nellen *et al.* created constitutively active forms of a Dpp receptor — in which the receptor is active even in the absence of Dpp — and expressed marked clones of this construct in the wing disc. The reasoning is that if cells express an activated Dpp receptor, they should think they have been exposed to Dpp, and if the response to Dpp is to set up a signalling relay, *omb* and *spalt* should be activated in distant cells. If cells exposed to Dpp do not set up a relay, the effects of the constitutively active Dpp receptor should be strictly cell-autonomous.

The Dpp protein is one of the transforming growth factor- $\beta$  (TGF- $\beta$ ) family of signalling molecules, and like its fellow family members it signals through a complex composed of type I and type II receptors. In *Drosophila* there are two type I receptors encoded by the products of the *thick veins* (*tkv*) and *saxophone* (*sax*) genes<sup>12-14</sup> and a type II receptor is encoded by *punt*<sup>15,16</sup>. Nellen *et al.* made several forms of constitutively active Dpp receptor, but the simplest involved making a single amino-acid change in *thick veins* to create the mutant TKV<sup>Q253D</sup>; the analogous mutation in a type I TGF- $\beta$  receptor is known to create a constitutively active receptor, and subsequent experiments demonstrated that ectopic expression of TKV<sup>Q253D</sup> in a wing imaginal disc causes activation of *omb* and *spalt*. ▶



When small clones of cells expressing TKV<sup>Q253D</sup> are generated in the imaginal disc, expression of *omb* and *spalt* is restricted exclusively to expressing cells: there is no long-range effect. So a relay model appears to be ruled out and a powerful blow is struck for diffusible morphogens. (Only the most critical of commentators would remark that it might also have been interesting to create clones of constitutively active Sax, in case it is saxophone-mediated signalling that is responsible for generating a relay!)

But — as always — it turns out that things are not so simple. In their paper on page 387, Lecuit and colleagues<sup>9</sup> describe experiments which agree with those of Nellen *et al.* in suggesting that Dpp is able to diffuse over long distances. Like Nellen *et al.* they show that the effects of TKV<sup>Q253D</sup> are strictly cell-autonomous; and they additionally demonstrate that clones of cells mutant for *Mothers against dpp*, a component of the Dpp signal-transduction pathway, fail to express *spalt*, even though they fall within the *spalt* expression domain. Both of these observations again constitute strong evidence against a relay model.

So far, so good. The first hint that things may be more complicated comes from an experiment in which Lecuit *et al.* expressed higher-than-normal levels of Dpp in its normal expression domain. Although, as reported by Nellen *et al.*, the *spalt* domain is enlarged in such imaginal discs, the *omb* domain (which Nellen *et al.* did not study) is not expanded, and it ends up the same width as that of *spalt*. This would not be predicted by the diffusible morphogen model, and nor would Lecuit and colleagues' next observation that, when marked clones of Dpp-expressing cells are generated, the surrounding halo of *omb* expression is slightly smaller than that of *spalt* — not, as the other group had found, larger.

What's going on here? Lecuit *et al.* explain their results by proposing that *omb* and *spalt* respond to similar concentrations of Dpp but that they then differ in the way they 'remember' exposure to the signal. This suggestion is based on work in which TKV<sup>Q253D</sup> is activated in the normal Dpp expression domain in a Dpp mutant background. This activation of TKV<sup>Q253D</sup> depends on local activity of hedgehog protein, so if cells move away from the midline, due to growth of the imaginal disc, TKV<sup>Q253D</sup> expression is downregulated.

In these experiments, Lecuit *et al.* find that expression of TKV<sup>Q253D</sup> in the Dpp domain causes both *spalt* and *omb* to be expressed in the imaginal disc, but that the domains of expression are different: *spalt* expression is restricted to midline cells that continue to express the constitutively active receptor, whereas *omb* expression also occurs in cells which used to express TKV<sup>Q253D</sup> but have now

moved away from the midline and switched it off.

Overall, this experiment suggests that expression of *spalt* requires continuous activation of the Dpp signal-transduction pathway, and if growth of the disc causes cells to move out of range of the Dpp signal, *spalt* is downregulated. By contrast, expression of *omb* persists even if cells are moved far away from the Dpp signal. This proposal, which incorporates elements of Gurdon's 'ratchet', would result in *spalt* always being expressed close to the Dpp source and, given sufficient growth, *omb* being expressed at a distance.

According to this view, the difference between the two groups' 'haloes' of *omb* and *spalt* expression might be due to more growth occurring in the imaginal discs of Nellen *et al.* than in those of Lecuit *et al.* This explanation is not consistent, however, with Nellen and colleagues' observation that late-generated clones of cells mutant for TKV do not express *omb*, a result which implies that *omb* expression requires direct and continuous exposure to Dpp. It is also at odds with their finding that ectopic expression of *dpp* under the control of a weak promoter can only activate expression of *omb*, and not *omb* and *spalt*.

These are significant issues. But the most important point in both papers remains — the fact that Dpp is able to act at a distance by direct action, thus pretty much ruling out a relay model. Nevertheless, we now need to ask whether differen-

tial gene expression in response to Dpp occurs primarily through concentration-dependent mechanisms or through a cellular memory. Future experiments might include a careful temporal study of *omb* and *spalt* gene expression in response to ectopic expression of TKV<sup>Q253D</sup> and Dpp. By monitoring growth and gene expression simultaneously, it should be possible to come to a final decision as to which of the two models better reflects reality. □

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## NEUROSCIENCE

# Cognitive cartography

Bruce McNaughton

CELLS in the rat hippocampus each fire when the rat is located in a specific region of the environment, called the cell's 'place field'. A population of place fields covers, more or less evenly, every portion of the environment, thereby forming a 'cognitive map'<sup>1</sup>. It is thought, then, that place cells are responsible for an animal's spatial awareness, and as such they are of great interest.

On page 425 of this issue<sup>2</sup>, O'Keefe and Burgess provide new insight into the mechanisms responsible for cognitive maps: they show that stretching the walls of a box in which rats have been trained causes a corresponding stretching or splitting of place fields, and suggest that this can be accounted for if each cell is tuned to a specific combination of distances from a subset of walls. But are hippocampal place cells simply visually driven distance detectors, sensitive to landmark configurations or environmental shape?

Consider what makes the problem of

place fields a difficult one. The most straightforward account would be that place fields result from each cell receiving a unique, complex combination of sensory inputs from the outside world. This 'local view' hypothesis explains a great deal, but, in the end, it fails to suffice. The difficulty is that most, if not all, external sensory input can be removed without seriously disrupting place fields. Normal place fields can occur in the complete absence of visual and auditory cues<sup>3</sup>, and olfactory cues have also been shown to be unnecessary<sup>4</sup>. Indeed, place fields can be established in complete darkness and persist without change when the lights are turned on<sup>5</sup>. Moreover, in symmetrical environments, their pattern may show spontaneous rotation<sup>6</sup>, even when distinct asymmetric visual cues are present.

Suppose we place a rat inside a large, high-walled cylinder, coloured homogeneously grey except for a white cue card covering 100° of arc. We ask the rat to

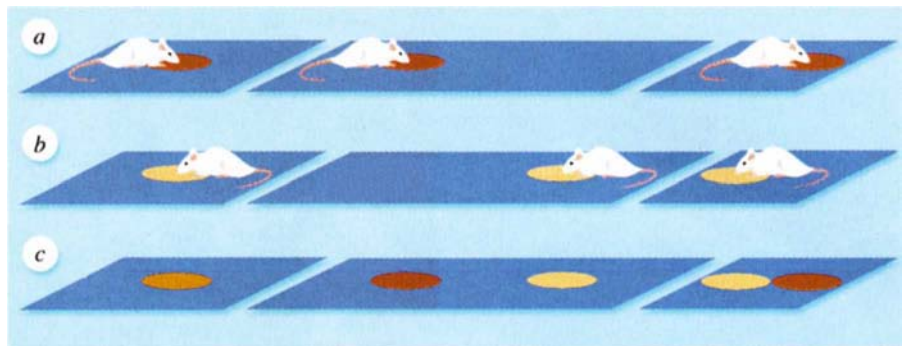
forage for randomly scattered bits of food; meanwhile we record the activity of a set of hippocampal place cells. After we have obtained an adequate sample, we remove the rat from the cylinder for a moment, rotate the white cue card by 90°, and then place the rat back inside. Most commonly, the result is that the place fields of all the cells rotate by the same angle as the cue card<sup>7</sup>. Suppose now that we turn out all the lights while the rat is inside the cylinder. The rat in this situation continues to forage busily for food, and the place cells continue to fire in essentially the same places they did before.

Suppose we now quietly rotate the cue card by 90°, in the dark, without notifying the rat. As we would expect, the place cells again continue to fire without disruption. Finally, we turn the lights back on, with the cue card now in a different location from the last time the rat has seen it. Often, the result of this sort of experiment is that the place fields rotate rapidly to match the cue card. Sometimes, however, this rotation is delayed or does not occur at all<sup>8</sup>. The bottom line is that external sensory cues — particularly visual cues — influence, but do not rigorously dictate, place-cell activity.

These properties have been understood for some time, but there is still no clear understanding of how individual sensory cues influence place cell activity. In most experiments (see for example ref. 8), manipulation of cues has resulted either in no apparent effect, or else in a complete reorganization of the place field array, as if the rat had adopted an entirely new map.

With the exception of Gothard *et al.*<sup>9</sup>, who found place cells bound, at fixed distances, to specific, behaviourally relevant landmarks, O'Keefe and Burgess<sup>2</sup> are the first to succeed in parcelling out the influence of distinct cues. They account for the stretching and splitting of place fields by assuming that each wall of the box makes an independent contribution to each place cell. When these contributions exceed a certain threshold, the cell begins to fire. Indeed, this hypothesis accounts for most of their data quite well. But there is a critical feature of the data, illustrated in Fig. 2c and d (page 426), which this hypothesis does not explain, and which leads to a very different interpretation of the origin and dynamics of place-cell firing.

The issue centres around how these cells calculate the distance from the boundaries. The obvious guess would be that they do so visually, but the data speak against this. As the authors report, when a place field splits, the activity in each half-field shows a strong dependence on the direction in which the rat is facing — but, strikingly, the wall behind the rat is the one that controls where the cell fires (that is, the wall the rat is coming from, rather than the wall it is moving



a, b, Simplified representation of the directionally biased splitting that occurs in hippocampal 'place fields' when rats are trained in a homogeneous square box and then placed in a similar box with one or two dimensions increased about twofold<sup>2</sup>. a, In such environments a given hippocampal cell normally fires in one region (the place field) independently of which direction the rat is travelling in. The vector of activity in a population of place cells provides a distributed representation of the rat's location. b, When the box was enlarged, O'Keefe and Burgess<sup>2</sup> observed place-field splitting, with each half-field now dependent on the direction the rat was travelling in. The position of each half-field was dependent on the distance to the wall from which the rat had come. The authors suggest that the normal, non-directional place field is a composite of several directional ones, but the phenomenon can be explained more simply on the basis that place cells are continuously updated by self-motion information relative to the last known location. In this case, it appears that the rats take position fixes when they get near to or contact a wall, and then measure distance from the wall by path integration ('counting' their footsteps). c, If the environment were to be compressed somewhat instead of enlarged, this second hypothesis predicts a splitting with the opposite directional dependence.

towards, and presumably looking at). This would seem to suggest that memory of some kind is involved.

One possible explanation, consistent with a variety of other evidence<sup>10</sup>, is that the rats in this situation take 'position fixes' when they are close to the walls, and then update their place cells primarily on the basis of path integration — that is, an intrinsic mechanism for updating the spatial coordinate based on information from the animal's own motion. In a stretched environment, this would lead to hysteresis in the place fields, with the observed directionality. A location co-ordinate in the cognitive map would be found at one physical location as the rat moved away from one wall, and at a different location as it moved away from the opposite wall.

A natural, and testable, prediction following from this explanation is that if the environment were to be compressed rather than expanded, hysteresis in the opposite direction would be observed (see figure). According to this view, place fields do not explicitly represent anything at all about the external world. They are merely a part of a population code for location relative to the place fields of other cells. The metric which establishes the physical distance relationship between two groups of place fields is the animal's own movement<sup>10</sup>, and, in many circumstances, place fields seem to be determined more by their distance relationships to each other than by external stimuli.

Self-motion signals are imprecise over long distances or times, and this would lead to the accumulation of position errors. When the activity of a group of place

cells is repeatedly associated with specific external stimuli, however, learning appears to take place. These stimuli subsequently provide the possibility of the rat's taking position fixes and correcting the accumulated errors. But in my view the place cells do not become 'detectors' for the associated stimuli as the authors imply.

In all, then, it appears that O'Keefe and Burgess's rats take their position fixes on contact with the walls of the apparatus, not by looking at them. According to this view, directionally biased place-field fission results not because place fields are "composed of several superimposed directional subcomponents"<sup>1</sup>, but because the rats in the experiment only take position fixes when they bump into the walls of the apparatus. Between the walls, they rely on path integration. □

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# Seamounts make earthquakes

Larry J. Ruff

IMAGINE a tremendous force pushing against the side of a huge mountain, so that the mountain is sheared off at its base. It may be difficult for us to comprehend the forces required for this mountain 'decapitation', but it is easy to believe that huge earthquakes might be caused by such an event. In their recent article published in *Geology*, Cloos and Shreve<sup>1</sup> speculate that shearing of seamounts buried in subduction zones causes many of the world's largest earthquakes.

Given the visual image of a decapitated mountain, Cloos and Shreve's speculation certainly has some intuitive appeal. Although the idea seems simple, the case for seamount decapitation causing large earthquakes draws upon diverse facts from subduction zone tectonics and global seismicity.

New oceanic lithosphere is created at mid-ocean ridges, and as it spreads and ages the sea floor gradually attains a typical depth of about five kilometres below sea level. Eventually, the lithosphere encounters a subduction zone, where it is forced beneath the wedge-shaped overlying plate (see figure). Sitting on top of the otherwise smooth sea floor are isolated mountains called seamounts. Their peaks are usually submerged, but their height above the sea floor can reach 4 km or so — they are big mountains.

So what happens to seamounts, or any other 'bumps', as the sea floor passes beneath the other plate? The pressure crushes any void below a depth of one kilometre, so either the bumps are all

sheared off at a shallow depth to make the top of the subducting sea floor smooth, or sediments fill in around them to provide a smooth surface.

Most of the world's large earthquakes occur in the subduction zones around the rim of the Pacific Ocean. The largest of these earthquakes are caused by sudden slips at the interface between the two plates, above a depth of about 60 km (ref. 2). The Pacific subduction zones generate many magnitude-seven events a year, and three events this century have been larger than magnitude nine.

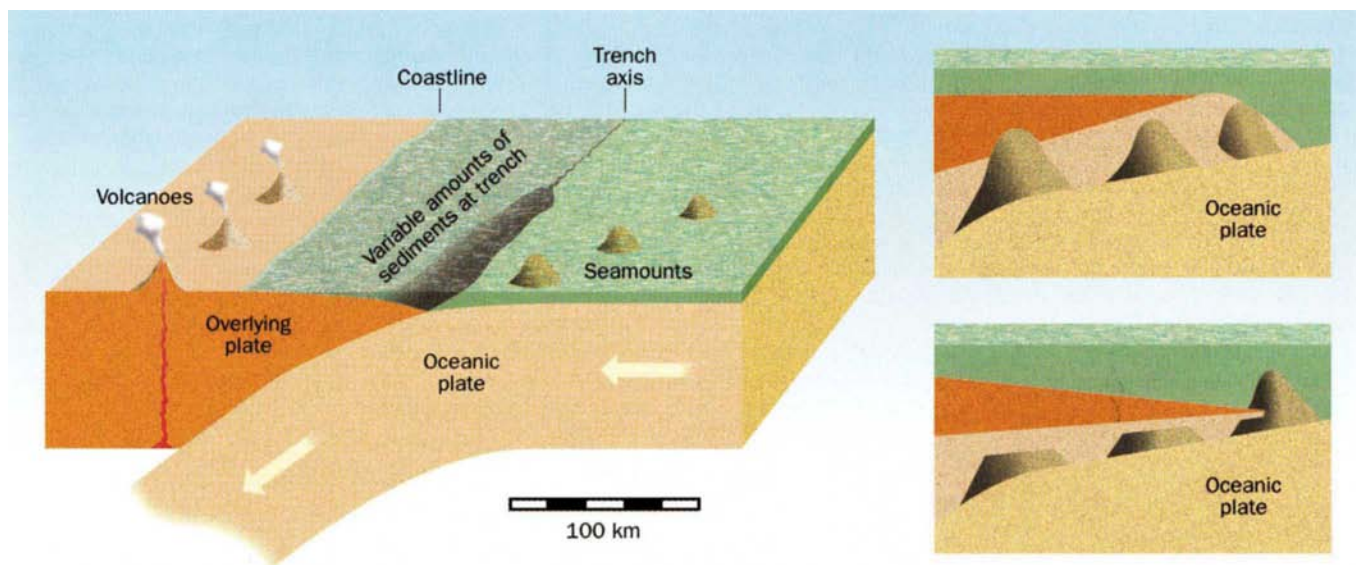
Seismologists have shown that larger earthquakes are caused by a larger fault area. For example, the greatest subduction earthquakes, of magnitude 8.5 or more, rupture fault areas that are 100 km wide and extend for several hundred kilometres along the subduction zone. The merely 'great' magnitude 8 events have rupture zones that are about 100 by 100 km, and 'modest' magnitude 7 events have fault areas that are about 20 km across. We also know that fault slip generally increases as the fault area increases — typical values range from 1 m to more than 10 m as earthquake size ranges from 7 to 9. So our visual image of instant mountain decapitation is a bit exaggerated. It takes one thousand magnitude-nine earthquakes to completely decapitate a typical seamount with a base 10 km wide.

In any one earthquake, the slip distance varies along the fault. One interpretation is that stronger portions of the plate interface slip further. These strong subregions

are called asperities<sup>3</sup>. Rupture of these asperities controls the place, the time and the fault area of the largest earthquakes. Asperities are a useful concept, but 'strong subregion' is a rather vague definition. Suggestions for their physical nature include irregularities in the subducted sea floor<sup>4</sup>, the overlying plate<sup>5</sup> or the interface itself<sup>6</sup>. Although we do not yet know what asperities are, we think they control the size of earthquakes!

Another key observation is that earthquake size varies between zones. Uyeda and Kanamori<sup>7</sup> noted that a few zones, such as the Chilean, consistently generate the largest earthquakes, while all other subduction zones, like the Marianas, generate smaller events. They speculated that this diversity is explained by the 'absolute' velocity of the overlying plate (relative to the deep mantle): Marianas-type zones result from the overlying plate moving back away from the oceanic plate, reducing the speed of subduction; Chilean-type zones have it charging over the trench. Scholz and Campos<sup>8</sup> have extended this idea. In their model, the subducted plate acts as a 'sea anchor' in the mantle 'ocean', and the resultant forces partly determine whether a particular subduction zone is a Chilean-type or Marianas-type. The connection between a global tectonic parameter, such as mantle flow, and plate interface asperities is not obvious.

In contrast, two local tectonic variables that may have a direct influence on asperities are sea floor roughness and the style of sediment subduction. We might expect that subduction of a 'rougher' sea floor with few sediments would result in larger earthquakes, but actually it produces smaller ones. The largest earthquakes occur in zones with tremendous



Two different ways a seamount can be subducted. Seamounts can cause small or large earthquakes depending on where they are decapitated, which depends on the amount of sediment at the trench. That in turn depends on the geometry of the 'subduction channel'. Top right: a narrow channel mouth scrapes off sediments and decapitates

seamounts at the trench, causing small earthquakes. Bottom right: a channel that starts wide and narrows with depth allows sediments to accumulate and seamounts to reach a depth of perhaps 40 km, where their decapitation may be responsible for great earthquakes.



piles of sediments at the trench<sup>4</sup>. Further, the sea floor that subducts beneath northern Japan changes from 'smooth' to 'rough' along the trench, and the variations in sea floor roughness directly correspond to the pattern of earthquake occurrence<sup>9</sup>. In particular, large earthquakes occur where 'smooth' sea floor is subducted. It seems that plentiful sediments and smooth sea floor result in large asperities<sup>1</sup>.

Cloos and Shreve<sup>1</sup> now offer a direct connection between a physical feature and the global variation in large earthquake occurrence. The idea is that subducted seamounts make the asperities that control earthquake occurrence, but that earthquake size depends on where the seamounts are decapitated, not, as in an earlier model, on their size<sup>10</sup>. If the seamounts are sheared off at the mouth of the trench, then only small earthquakes are produced. But if they are first subducted down to 40 km or so, the decapitation produces large to great earthquakes.

The amount of sediment present at the trench is connected to the geometry of the 'cutting blade' of the overlying plate (see figure). Seamounts that protrude from the sediment are decapitated at the trench, resulting in small earthquakes. There are no large earthquakes further down dip because the 'subduction channel' is filled with the rubble of sediments and sheared-off bumps, and this rubble does not make large asperities.

The other extreme is where the seamounts are covered by sediment, and easily subduct through the shallow part, but eventually encounter the roof of the overlying plate down at a depth of about 40 km. The seamount then becomes an asperity. Since great earthquakes require fault zones more than 100 km across, Cloos and Shreve suggest that the seamount asperity initiates the rupture, which then spreads over a much larger area into strong, metamorphosed sediments.

This idea seems to satisfy several observations about great earthquake occurrence. Of course, it must now be tested with new observations. The seismological literature is littered with intuitively appealing explanations that could not sur-

vive the grim reaper of closer scrutiny. But for the moment we can explain some aspects of earthquake occurrence with the following subduction tale: seamounts try to hide in sediments to escape the blade of the overlying plate, but they all lose their heads eventually. Those that are decapitated quickly, at the trench, do so with the

minor complaints of small earthquakes, but those that evade decapitation for some time protest about their fate with the booms of great earthquakes. □

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## ECOLOGY

# Invertebrates and mycophagy

Peter D. Moore

THERE is nothing very remarkable about eating fungi, although it does make life difficult for those who enjoy constructing diagrams of food webs and wish to assign organisms to neat trophic levels. Various animals from flies to reindeer take culinary delight in certain toadstools and some, such as termites and leaf-cutter ants, actually farm fungi by supplying

branching and the generation of staphylae is stimulated.

They tested the efficacy of ant care on fungal productivity by growing samples of fungus garden in petri dishes and exposing some of them to ant activity for just three hours, while control samples were isolated from the ants. The numbers of staphylae in each dish were counted and, predictably, significantly fewer staphylae survived in those samples exposed to the depredations of ants. Two days later, however, the situation was reversed and ant-exposed samples had 30 per cent more staphylae than the controls, so the attentions of the ants had generated a higher density of the swollen hyphal bunches.

The question arises whether the stimulation observed was simply a consequence of mechanical damage and resulting mycelial branching, or whether some chemical exudate is involved, as has been proposed for stimulation of herbaceous growth by mammalian grazers<sup>3</sup>. To test these options four experiments were set up, in which staphylae were harvested artificially using a needle; hyphae were stroked and broken with a needle; ant faeces were applied without causing physical damage; and the crushed heads of workers, suspended in water, were added to the culture (with the idea of simulating the effect of exudates from ant labial glands).

The first two treatments initially reduced the numbers of staphylae but after three days these gardens had significantly greater crops (including the harvested material) than the samples treated with ant faeces, head extracts or the untreated control. The conclusion, therefore, is that the mechanical pruning activity of the ants stimulates the fungus into producing new staphylae. This does not, however, preclude the possibility that some other gardening activities, such as the control of invasive fungi, may involve chemical treatments by ant exudates.

The process described here is entirely vegetative on the part of the fungus; no change in resource allocation to reproductive structures is involved. As the authors point out, this response is unlike that involved in the human pruning of fruit trees, where resources are channelled from



M. Bass

Down on the fungal farm — a worker ant consumes a staphylococcus.

them with a plant-based energy resource to ensure their productive growth. But the observations of M. Bass and J. M. Cherrett, published in the latest issue of *Functional Ecology*<sup>1</sup>, show that the horticultural activities of some mycophagous ants even extend to pruning hyphae as a growth stimulant, and this adds a new dimension to an already remarkable story of invertebrate agriculture.

Leaf-cutter ants gather fresh leaf material as a substrate for the cultivation of basidiomycete fungi which they cultivate within their nests. These fungi do not fruit but produce bunches of swollen hyphae (staphylae) that are used by the ants and their larvae as food, being relatively rich sources of energy and having a reasonable content of nitrogen and other nutrient elements<sup>2</sup>. Many ant workers spend time licking the hyphae, in the process of which some are broken and ingested by the ants, and Bass and Cherrett came up with the hypothesis that this activity constitutes a pruning process during which mycelial

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herbaceous to fruit production. It is, however, comparable to the mowing of grass where mechanical damage stimulates vegetative proliferation.

Ants are not the only mycophagous group among the invertebrates, and a report by Bultman and Mathews in *Oikos*<sup>4</sup> describes the fungal forays of a millipede, *Aniulus bollmani*, as it seeks out fruiting bodies of an ascomycete fungus that lives on the stalks of various grasses. But no fungus story, it seems, is ever simple. In this case the fungus, *Epichloe typhina*, has a mutualistic relationship with a fly, *Phorbia phrenione*, which is responsible for transferring gametes from one strain of the fungus to another in a process analogous to pollination<sup>5</sup>. Once fertilized in this way, *Epichloe* produces ascocarps (fruiting bodies) within which ascospores are formed. The fly, of course, receives its pay-off by laying its eggs in the fungal stroma and the larvae feed on developing ascocarps. At this point the millipede enters the picture in its random peregrinations up and down grass stalks. Risking drought and predation in its quest, it consumes fungal fruiting bodies wherever it comes across them.

Bultman and Mathews have been able to demonstrate that the millipede is a very effective predator of the fungus despite its apparent inability to locate infected stalks from its lowly position on the ground (the fungal fruiting bodies are usually 10–20 cm up). Although only about 20 per cent of grass stalks were infected by the fungus in the study sites (in northern Missouri), a three-year survey of specific infections showed that the millipede succeeded in locating and damaging 45 per cent of them. The damage overall was greater at the ascocarp phase than at the sexual, spermatial phase, and the preferred time for consumption of the ascocarps was before the spores were released.

The authors very reasonably conclude that the influence of the millipede is essentially negative as far as the fungus (and probably the fly) is concerned. The millipede is clearly a cheat, muscling in on a comfortable mutualism between a fungus and a fly. But could there be another twist in the tale? What are the mycelial growth consequences of millipede grazing? Does the fungus benefit from covert pruning? Or is the millipede gut acting as an unwitting transport system for dispersing ascospores? Clearly, one should never underestimate millipedes or moulds. □

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## Continents of the core

Michael E. Wyssession

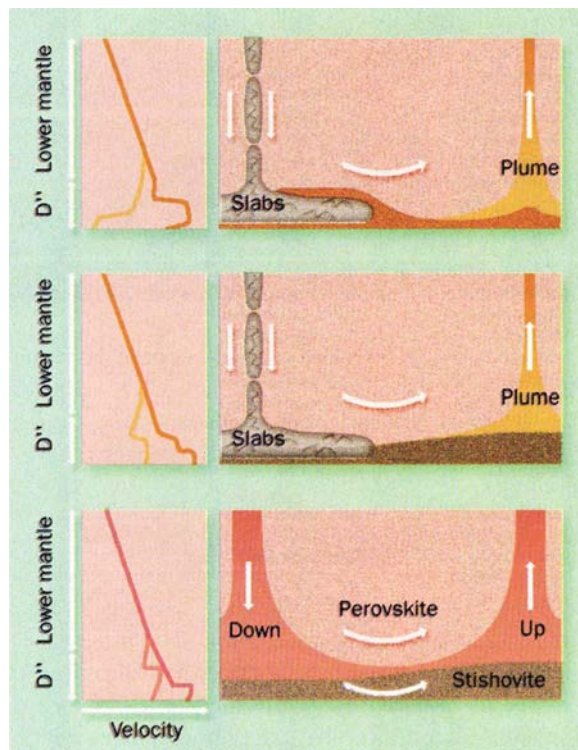
THERE is a lot more going on at the core–mantle boundary, 2,900 km below the surface of the Earth, than one might gather from memories of clean, cut-away diagrams in textbooks. Immediately above the core lies a 200 km thermal boundary layer called D'', where the properties of the mantle change. The characteristics of D'' are already known to vary around the globe, and now, on page 409 of this issue<sup>1</sup>, Kendall and Silver report a high degree of seismic anisotropy within the layer. To interpret the cause of this behaviour we must consider all the other seismic properties of D''.

If you look at all the geological processes occurring at the surface of the Earth, and then imagine how they would work upside-down, at 1.36 million atmospheres of pressure and at 3,500 K, you might begin to get a sense of what is occurring at the core–mantle boundary (CMB). There are likely to be chemical and thermal boundary layers analogous to the crust and lithosphere; there is evidence of continent-sized variations in seismic velocity, as well as much smaller features which scatter high-frequency seismic waves; there is an inverted topography on the silicate mantle as it sticks into the liquid outer core; and there is a sudden large increase in seismic velocity 200–300 km above the CMB that varies in height, and in some places is not even observed — it is even possible that there may be more than one discontinuity in some places. The velocities of seismic waves in D'' vary horizontally by several per cent, comparable only to Earth's surface. And, as at the surface, they do not always vary in tandem, implying chemical or phase variations rather than just changes in temperature. In some regions there is an ultra-slow velocity layer at the very bottom of D''.

There has been no lack of attempts to understand the base of the mantle, and speculations still outnumber observations. D'',

or parts of it, has been called the "graveyard of subducted slabs" (slabs of oceanic crust and lithosphere), the origin of hotspots, the accumulation of mantle dregs, the product of mantle–core chemical reactions, a zone of partial melt, a mineralogical phase transition, a layer of delaminated oceanic crust, and the defining constraint for outer core flow. Not all of these can be correct... or maybe they can.

Anisotropy in D'' has been observed in shear waves that diffract around the core, with vertically vibrating waves trailing behind horizontally vibrating waves by up to 9 seconds<sup>2–4</sup>. This should not come as a surprise. Seismic anisotropy occurs in the crust, the upper mantle and in the inner



Three models of different chemical and physical structures that could explain some characteristics of the lowermost mantle. The left-hand figures show the corresponding seismic shear-wave velocities in the region of downwelling (darker) and upwelling (paler), for the lower mantle and the D'' transition layer. a, Subducted slabs of oceanic crust (thick black lines) and lithosphere accumulate in D'', with their low temperatures causing the discontinuous velocity increase at the top of D'', and partial melting of the crust causing seismic transverse anisotropy. b, Again slabs accumulate in D'', but here the sudden velocity increase is caused by an accumulation of high-density post-eclogite oceanic crust. Convection in the overlying mantle sweeps up this chemical boundary layer, causing variations in thickness. An additional mechanism is required for anisotropy. c, The breakdown of perovskite into stishovite and magnesiowüstite causes the velocity discontinuity. The discontinuity is ubiquitous, and variations in D'' thickness are variations in the depth of the phase transition in response to lateral thermal and/or chemical variations. Anisotropy is provided by the structure of the stishovite minerals. Slab accumulation might still occur but is not required.

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core, but for different reasons in each case. In the crust it is largely due to the layered rock fabric, whereas the upper mantle and inner core are mineralogically anisotropic owing to the asymmetric crystal structure of olivine, whose small crystals are partially ordered in the upper mantle by convective stresses, and hexagonal-close-packed iron, which may form a single crystal of the whole inner core. So is anisotropy in D" the result of layering or mineralogical asymmetry? The answer is not so clear.

Kendall and Silver propose a layering mechanism in the 'slab graveyard' picture for the anisotropy they observe, as shown in part *a* of the figure. Remnants of oceanic crust accumulate and form horizontal layers of partial melt, slowing down vertical shear waves. The slabs should still be cold enough to be anomalously fast, explaining the overall velocity increase. This makes sense, because D" beneath the Caribbean corresponds to the projected location of the palaeo-Farallon slab<sup>5</sup>, which subducted beneath western North America during the Mesozoic (225 to 65 million years ago).

Can the sudden velocity increase of up to 3 per cent seen at the top of D" be caused thermally? Possibly not, because slabs at the base of the mantle would have had a long time to approach thermal equilibrium, and even without partial melt would be only a few hundred degrees cooler than their surroundings. But if the high-temperature, low-viscosity bottom part of D" can be displaced over the cold upper-mantle rock pooling on the core, such a velocity jump would be possible.

We would also like to explain the anisotropy beneath the eastern Pacific Ocean, a region of anomalously slow velocities, with no expected slab material. Yet anisotropy of the same style and magnitude may also be present there<sup>3,4</sup>. Could something else act in the same way as partial melt of post-eclogite rock from oceanic slabs does in the Caribbean?

It is also unclear why the melt should form horizontal layers, rather than being mixed up in a complicated mélange. Estimates of melting temperatures for silicate phases at CMB pressures can vary by more than 1,000 °C, and we do not know that the rock should even be molten. In fact, other studies have called on a solid layer of post-eclogite as the cause of the discontinuous increase in D" velocities<sup>6</sup> (part *b* of the figure).

If the anisotropy in D" were of mineralogical origins, a suitable mineral is the high-pressure phase of SiO<sub>2</sub>, stishovite, which may undergo a phase change to a CaCl<sub>2</sub> structure that would make it highly anisotropic<sup>7</sup>. In fact, at the theoretical limit, the shear velocity in one direction would drop to zero. The lower mantle is mostly perovskite, and it is possible that the discontinuous velocity increase we see at the top of D" corresponds to the insta-

bility of perovskite. At this point perovskite might break down into stishovite and magnesio-wüstite, and the result would be an anisotropic increase in velocity (part *c* of the figure). However, this would require the stability field of perovskite to co-incidentally end at the same depth as the CMB boundary layer or for some other material to undergo a yet-to-be-observed phase transformation, and is no less speculative than the partial melt scenario.

Our understanding of the melting points and physical behaviours of lower mantle minerals is still too poor to be able to select between different models of D", but we are beginning to identify the key questions. Is the D" discontinuity a ubiquitous feature? What causes its lateral change in thickness? What is the origin of

#### PALAEOCLIMATOLOGY

## A la recherche du temps perdu

Phil Jones

OCEANIA covers a quarter of the Southern Hemisphere, encompassing Australia, New Zealand and the South Pacific, but the vast records of past climate change the area holds have remained largely unexplored. Two workshops\* have helped to change that.

Only recently have temperature and precipitation data from the past 150 years been brought together and rigorous analyses begun<sup>1</sup>. Long-term warming is evident in New Zealand (1.1 °C since the 1890s) and Australia (0.7 °C since 1908), but the islands north of the South Pacific Convergence Zone only began warming in the late 1970s. This recent warming may be related to the increasing frequency of El Niño events.

In contrast, few long-timescale trends are evident in precipitation. There is considerable decade-to-decade variability evident, related to changes in the strength of El Niño. Parts of the region show intriguing changes in the interrelationships between El Niño and temperature and precipitation around 1971–72, probably because of an increase in cloudiness. For example, precipitation is now more strongly related to El Niño than it was before 1970. Two climate data sets released on CD should aid the study of these relationships between surface climate and circulation (Neville Nicholls, BMRC Melbourne and Rob Allan, DAR, CSIRO, Aspendale).

\*Australian Tree Rings, Ice Corals and Corals, Aspendale, Australia, 13–15 February 1996; and Climate Trends in Oceania since AD 1500, Auckland, New Zealand, 15–17 April 1996.

the ultra-slow layer at the bottom of D"? At what scales does horizontal seismic velocity vary? And what is the cause of D' anisotropy? Only by simultaneously satisfying all of the clues to this enigmatic region can we solve the puzzle of the CMB, Earth's other surface. □

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Oceania has a wealth of natural archives of the past, and in each of the three main areas (tree rings, corals and ice records) pioneering work is being undertaken.

A tree-ring chronology going back to 1723 BC has been developed in Tasmania, using high-elevation, subalpine Huon pine. These trees respond to summer tempera-

IMAGE  
UNAVAILABLE  
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REASONS

A black coral of the New Zealand fiordland, with a yellow starfish coiled near the centre. The annual growth rings of this coral might be used as a record of past climate, as tree-rings have been used elsewhere.

ture, and show that since the 1950s temperatures have been warmer than at any time since the start of the record<sup>2</sup>, except for a short period around 300 BC. Surprisingly, the lowest temperatures overall occurred in the late nineteenth century, so the full range of variability seen in Tasmania over the past 3,700 years<sup>3</sup> is seen within the brief instrumental period.

At lower elevations, sub-fossil samples of the same material may yield a chronology back to the late Pleistocene, extending the radiocarbon calibration curve back to about 30,000 years ago, and enabling more accurate dating of late glacial material and the measurement



of changes in solar variability.

Another biological record is found in corals. The greatest expanse of tropical corals anywhere in the world occurs on the Great Barrier Reef, but, compared with dendroclimatology, coral sampling is in its infancy. Much work is needed to understand how and why coral skeletons record information about their environment<sup>4</sup>. Despite this, sea surface temperatures have been reconstructed from coral growth rates back to the 1740s for Queensland coastal waters, and runoff from the Burdekin River—a good measure of precipitation in Queensland—has been measured back to the 1640s from coral fluorescence<sup>5</sup>.

South Pacific records are, except in the Galapagos Islands<sup>6</sup>, much shorter than those of the Great Barrier Reef. Further south, the temperate black corals of the New Zealand fiordland grow in rings, more like underwater trees than the massive corals of the tropical regions, but neither a cross-dating system nor any relationship with climate has yet been determined.

The last disciplines discussed at the two meetings were ice cores and glaciology. The Australians have extracted several ice cores from the Law Dome in the Antarctic, a location that is unique. Fresh snow produces up to 1.2 metres of ice a year, and there is no summer melting, so annual resolution is possible back to the early Holocene (8,000 years ago). The deepest ice (1,200 m) is more than 50,000 years old<sup>7</sup>, and the cores provide the most detailed history yet constructed of global atmospheric composition and local seasonal temperatures for this period.

The records show the industrial CO<sub>2</sub> increase of the last century, modulated by El Niño events. They also show that a drop in the CO<sub>2</sub> concentration by about 10 parts per million occurred quite rapidly in the late sixteenth century<sup>7</sup>, possibly related to a temperature decrease in the Northern Hemisphere<sup>8</sup>, the 'little ice age'. That famous cool period does not appear to have happened in the Southern Hemisphere; in fact, the Law Dome oxygen-

isotope temperature reconstruction shows that the coolest time since 1300 was during the autumns and winters of the early 1800s (ref. 9).

The only temperate glaciers in the region occur in the Southern Alps on the South Island of New Zealand. Most glaciers were at their furthest extents when the first Europeans arrived, and it has become clear that their retreat since then has been dramatic: 35 per cent of the mass of the glaciers has been lost, amounting to 35 km<sup>3</sup> of water. A temperature rise of 1 °C since the 1850s, as shown by the instrumental record, explains the retreat well. Since 1980, however, changes in circulation pat-

terns (with more southwesterly winds, due perhaps to more frequent El Niño events) have led to a small re-advance.

Research in Oceania is important not only because it fills a gap in our global knowledge, but also because of the uniqueness of some of the records and the minimal impact of mankind on the environment. The eventual synthesis of data may provide alternative views of the recent past—views currently dominated by records from the North Atlantic. ▲

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## CONDENSED MATTER

# Thinly veiled promise

*Stephen D. Kevan*

THERE is a strong human predilection towards objects of high symmetry. The Hope diamond, for example, was comparatively worthless before it was cut into a highly symmetrical shape. In contrast, the concept of broken symmetry permeates much of modern physics, from particle physics and Big Bang cosmology to superconductivity and magnetism. The article by Carpinelli and colleagues on page 398 of this issue<sup>1</sup> shows that such issues are important in the context of surfaces and interfaces as well. The authors see direct evidence for a transition to charge density wave behaviour in ultra-thin films of lead adsorbed onto germanium. The most notable aspect of this achievement is that the structural transition is accompanied by a metal-nonmetal transition. For this system, the sensitivity of electronic properties to structure is particularly acute.

The forces that govern the symmetry and structure of a particular surface, whether clean or with adsorbed particles, have provided an enduring focus for many years. This issue has gained particular prominence within the context of the exotic properties of low-dimensional materials. The cuprate superconductors, for example, show quasi-two-dimensional properties that are thought to lead to their high superconducting transition temperatures as well as their unusual normal-state properties. Will it be possible to tailor surface and interface properties so that specific, desired electronic characteristics can be produced? The work by Carpinelli and colleagues provides an important first step in this direction.

A charge density wave is a type of electronic instability driven by the inherent degeneracy of the highest occupied electronic level in metals—the Fermi level<sup>2,3</sup>. It is a generalization of a Jahn-Teller distortion, which is observed in molecular systems when the highest occupied molec-

ular orbit is degenerate but not fully occupied. In a static Jahn-Teller distortion, the system can and does lower its total energy, electronic plus elastic, by breaking its structural symmetry and so splitting the degeneracy, allowing an electron to occupy a lower-energy state. The electronic energy decreases linearly in the distortion, whereas the elastic energy increases quadratically.

In materials that can produce charge density waves, electron degeneracy occurs over regions near the Fermi surface (the contour in wave-vector space that delineates the highest energy occupied levels in a metal). The instability comes from mixing of states with different wave vectors, which produces a standing wave of well defined periodicity. This is the so-called charge density wave.

Normally associated with the wave is a periodic lattice distortion—the ions are forced to lower their symmetry in response to the electronic instability. The wavelength of the charge density wave need not be related to the lattice spacing by a rational factor, but its periodicity is well defined over large distances and the electron states can still be labelled with well defined wave vectors.

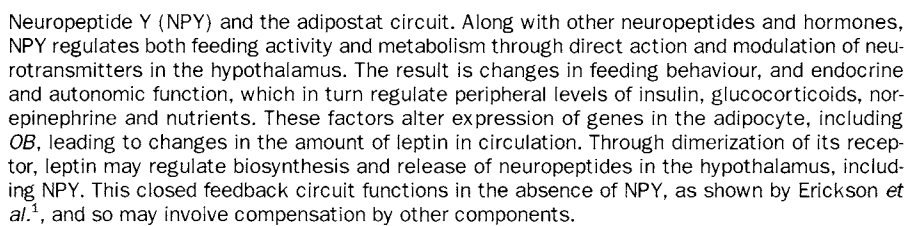
A Jahn-Teller distortion always produces a gap between the highest occupied and the lowest unoccupied levels, making the material an insulator. The same is not necessarily true of a charge density wave transition in a two-dimensional system. Depending on the shape of the Fermi surface and the strength of the coupling between different electronic levels, the distorted system may or may not have a band gap.

The aspect of Carpinelli and colleagues' work that is most surprising and that will probably generate the most interest is that the lead-germanium system apparently becomes non-metallic in the low temperature, distorted phase. This condi-

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The adipostat responds to a peripheral signal, leptin, that circulates at blood levels closely related to the amount of fat stores and mediates changes in the central regulatory circuitry of the hypothalamus, including NPY. In 1994, the gene encoding leptin was cloned from the genetically obese *ob/ob* mouse<sup>2</sup>, and subsequent studies appear to confirm its role in modulation of adiposity, as outlined in the figure. But regulation of feeding behaviour in the hypothalamus



In part, leptin acts by inhibition of NPY biosynthesis and release<sup>3,4</sup>, that action being mediated by a receptor that occurs in alternatively spliced forms as products of a gene responsible for obesity in the *db/db* mouse<sup>5</sup>. The long form of this receptor, including a cytosolic do-

main, has been found only in the hypothalamus. NPY is the most abundant neuropeptide in the brain, and is a member of a family of proteins, including pancreatic polypeptide (PP), peptide YY (PYY) and semaphorin, that are characterized by a proline helix folded onto an  $\alpha$ -helix (pancreatic polypeptide fold) with a carboxy-terminal tyrosine. The region of the hypothalamus involved in feeding has been reported to have the Y1, Y2, PP1 and perhaps a unique 'feeding' receptor. These receptors and peptides can cross-react, implying that the other peptides could replace NPY.

Physiologically, NPY seems to have various functions<sup>6,7</sup> — including involvement in regulation of circadian rhythm, anxiety and stress response, sexual function, peripheral vascular resistance and cardiac contractility, as well as of food intake and carbohydrate preference, and metabolic and lipogenic rate. Various lines of evidence show that NPY is the most effective neuropeptide in inducing feeding behaviour. Levels of the peptide and its messenger RNA correlate with the normal feeding cycle in rodents, and NPY neurons occur in the appropriate areas of the hypothalamus<sup>8,9</sup>. When injected centrally, antibodies and antisense oligonucleotides directed against NPY block normal onset of feeding. And NPY is the only known peptide that can induce obesity through prolonged central administration.

Erickson *et al.*<sup>1</sup> have produced an important animal model for studying the role of NPY in the long-term regulation of feeding and adiposity. In contrast to the acute intervention studies cited above, they report that the onset of feeding is normal following fasting in  $-/-$  mice. The response to leptin is intact and even more pronounced than in  $+/+$  mice: leptin promotes an exaggerated and immediate inhibition of food intake in  $-/-$  mice that tends to abate with time, whereas the response in  $+/+$  animals is slow, gradually increasing for days. Is the response to leptin in  $-/-$  mice physiological or the result of abnormalities arising from NPY deficiency? How does feeding behaviour and metabolism in  $-/-$  animals respond to nutrient excess, nutrient deficit or leptin administration for a time sufficient to alter their adipos-

ity? Cross-breeding with mice having known defects in body-weight regulation may give clues to the answers to these questions.

The release of NPY in the hippocampus has been referred to as that of an 'endogenous anticonvulsant' because of its inhibition of presynaptic excitatory glutamate release<sup>10</sup>. This inhibition is mediated by presynaptic Y2 receptors, which appear to be the predominant site for NPY action in this region of the brain. So Erickson and colleagues' observation of the propensity for  $-/-$  mice to have seizures, when under mild stress or after administration of an antagonist to the inhibitory neurotransmitter GABA ( $\gamma$ -aminobutyric acid), is an important confirmation that NPY participates in the inhibition of hippocampal glutamate release. Studies of other behavioural abnormalities expected with the  $-/-$  mutation could lead to further insight into NPY regulation of neurotransmitter release.

Finally, what about the question posed at the outset? Does the lack of observed abnormality in feeding in  $-/-$  mice indicate the existence of compensatory mechanisms, or does NPY play a more complex part in the regulation of feeding than we think? Targeted disruption of a member of a family of proteins can indeed result in compensation by other members<sup>11,12</sup>. Knockout of several brain proteins results in different phenotypes from those predicted from classical pharmacology, and immunolocalization, antisense oligonucleotide, and immunoneutralization studies<sup>13-17</sup>. These knockouts reveal subtle abnormalities that are at odds with the hypothesized role of NPY based on acute intervention studies.

So NPY may be one component in a rather plastic circuitry that features redundant molecular mechanisms and can withstand the loss of some of them. Selective knockout of NPY in specific locations of the hypothalamus, perhaps with genes that are upregulated in the mature animal, will probably be required to further our knowledge of the physiological action of NPY. □

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## Film for ever!

THE modern information age will disappoint historians. Our paper, made from acid wood-pulp, will decay in less than a century; our magnetic disks and cassette tapes will embrittle and peel, even if ceaseless changes of format leave any machines capable of playing them; the dyes in our colour films and photographs will fade to a drab beige. Only monochrome photographs, their metallic silver image stably held in cross-linked gelatine, will survive. Most of our so-called information is worthless trivia and is best forgotten anyway; but Daedalus would like to save something from its wreckage. He is devising an archival digital colour film.

Current colour films are 'analogue subtractive' systems. Incident light penetrates a series of coloured layers, each of which absorbs and subtracts a specific waveband. By contrast, a shadow-mask television tube is 'digital additive'. Red, green and blue dots of phosphor form a hexagonal matrix on the screen, are addressed as pixels by the electron beams, and their light output is added together.

At first Daedalus hoped to copy this additive architecture by printing a pattern of red, green and blue filter dots on a monochrome emulsion. Blue light (say) would activate the halide grains under the blue dots. Processing would block these dots with black developed silver, leaving the red and green dots free to transmit their sum — yellow, the complementary hue of the colour-negative.

But a true digital film should address the tiny halide grains as individual pixels. So Daedalus must line them up into an addressable array. He will exploit the fact that silver halides are diamagnetic. In a strong magnetic field, their grains repel each other. Expose the emulsion to such a field while it is still liquid, and its grains will adopt the arrangement of maximum repulsion — a perfect hexagonal array. The pattern of coloured dots, defining red-, green- and blue-sensitive grains, can then be printed on top in register.

Of course, the dyes in those dots will be just as fugitive as the ones in conventional film. Digital negatives and prints will fade as surely, but will leave a stable silver monochrome image behind. Furthermore, each grain will be digitally colour-coded by its position in the original matrix. A historian with a microscope could identify red, green and blue grains, and reconstruct the coloured image. Alone among our media, digital colour film will survive. Our age will shine down the centuries in all its vacuous chromatic splendour, but with no word of explanation.

David Jones

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## Dioxins in diesel exhaust

**SIR** — Particulates (mainly soot) emitted from diesel engines can be controlled by inserting a ceramic 'regenerative trap' or filter into the exhaust pipe. The filter is cleaned (regenerated) when the carbonaceous deposit ignites and burns away. In real engines, ignition must be promoted either by heating (electrically or by injecting a slug of fuel) or by catalytically burning the deposit. This is achieved by adding an organic metal compound<sup>1</sup>, for example copper, to the fuel. It decreases the quantity of soot formed in the engine and lowers the ignition temperature<sup>2</sup>. We show here, however, that particulate (soot) generation is ameliorated at the expense of increased dioxin emissions.

In the presence of a chlorine donor, dioxins/furans (PCDD/PCDF) can be formed by *de novo* synthesis in combustion gases as they cool through the range 250–400 °C (ref. 3). Diesel fuels contain small amounts of chlorine, which is known to form dioxins<sup>4</sup>. Certain metals can act as catalysts in these reformation reactions, copper being the most potent<sup>5</sup>. This raises the possibility that dioxin formation will be increased in a particulate trap system using copper additive in the fuel. To examine this possibility, we performed a series of tests using a small diesel engine driving an electrical generator, operated under nor-

mal conditions and when fitted with a regenerative soot trap.

We fitted a single-cylinder, 2.0-litre, indirect injection engine, rated at 3.5 kW nominal output with a cylindrical, commercial-grade cordierite monolith trap (Corning EX 66–100/17). The stock diesel fuel contained 0.9 p.p.m. chlorine as chloride. We prepared doped fuel by mixing 34.4 mg l<sup>-1</sup> of copper into the stock fuel. We tested for emissions at various engine loads but constant speed using the stock fuel without the soot trap, and the copper-doped fuel with the soot trap.

The exhaust gases were withdrawn isokinetically through a probe, directed through the trap and into a US EPA method 23 sampling train, modified method 5 (ref. 6). The organic compounds were adsorbed onto specially purified XAD-2 resin which had been previously spiked with a range of stable isotope PCDD and PCDF surrogate standards. We weighed the particulates from the probe, filter, resin cartridge, trap and impinger. The contents from the sample train were extracted with organic solvents, purified and analysed by high-resolution gas chromatography and high-resolution electron impact mass spectrometry.

The figure (a) shows the particulate

loadings of the exhaust gases, recorded as grams per litre of fuel used. The loadings with standard fuel increase by a factor of nearly three over the range of engine output. With copper present, the comparable particulate concentrations fall by 25 to 50%, with better results at higher outputs. This is consistent with the recorded behaviour of doped fuel<sup>1</sup>.

The dioxin/furan results are reported on a toxic equivalent basis (I-TEQ) to allow for the different toxicities of the congeners (see *b* in the figure). Using the doped fuel, there is a significant increase in dioxin emissions, especially at low load. There are therefore conflicting environmental effects from the addition of copper to diesel fuels.

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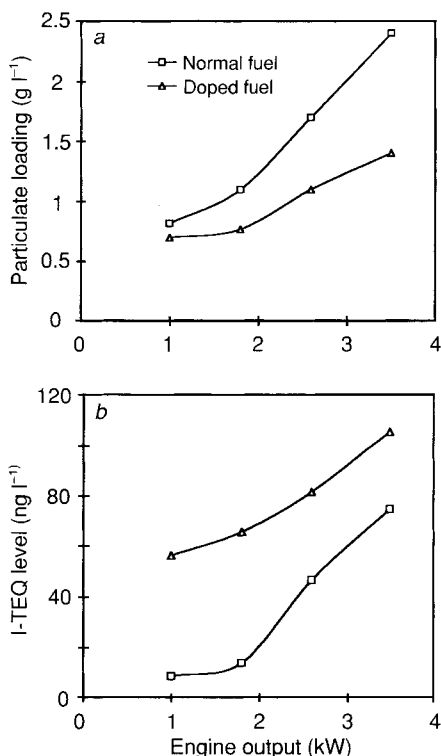
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a, Particulate formation at different engine loads; b, PCDD/PCDF formation at different engine loads.

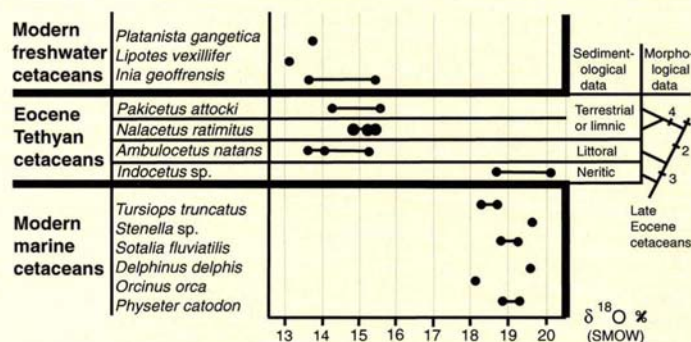
## Evolution of cetacean osmoregulation

**SIR** — Cetaceans (whales, dolphins and porpoises) underwent dramatic changes during their evolutionary transformation from four-footed land animals to obligate swimmers. The morphological aspects of this transition have only recently been documented with fossils<sup>1,2</sup> and provide a striking example of adaptation to a new environment. However, equally remarkable changes must have occurred in aspects of cetacean biology that are not reflected in the gross morphology of fossils. Here, we use oxygen isotope evidence to document the early presence in cetaceans of one of the most extraordinary physiological adaptations to life in the sea: their ability to survive without fresh water. This adaptation made it possible for cetaceans to live offshore early in their evolution and allowed them to disperse across open oceans, sparking a worldwide radiation in the Eocene.

Most mammals cannot survive without a freshwater source, and marine mammals have adopted various osmoregulatory strategies to cope with the salt in their environment. In contrast to some other marine mammals<sup>3</sup>, cetaceans<sup>4,5</sup> do ingest sea water, but it is unclear when the cetacean osmoregulatory system adapted to the excess salt

load associated with ingesting sea water.

To address this problem, we determined the oxygen isotope composition of phosphate in teeth of early cetaceans. Biogenic phosphate, particularly tooth enamel, appears to be highly retentive of its original oxygen isotope ratio under moderate diagenetic conditions<sup>6</sup>. Mammalian teeth are mineralized in isotopic equilibrium with body water, the isotope composition of which is dominated by ingested water<sup>7</sup>. Thus, the oxygen isotope composition of bone and teeth predominantly reflects that of ingested water. As fresh waters are isotopically lighter than sea water, tooth phosphate of mammals ingesting fresh water will have lower  $\delta^{18}\text{O}$  values than the teeth of mammals ingesting sea water<sup>8</sup>. Thus, the isotope composition of the teeth is a tracer of the broad categories (marine versus fresh) of water ingested. Factors in addition to the flux of ingested water may also influence oxygen isotope compositions<sup>9,10</sup>. We therefore tested whether ingested water dominates the isotope composition in cetaceans by analysing modern freshwater and marine species with a wide variety of diets and from a broad geographic range (see figure). The magnitude and direction of the



Oxygen isotope compositions of tooth phosphates of cetaceans. Isotope compositions are expressed relative to standard mean ocean water (SMOW) as  $\delta^{18}\text{O}_\text{P} = [({}^{18}\text{O}/{}^{16}\text{O})_{\text{sample}}/({}^{18}\text{O}/{}^{16}\text{O})_{\text{SMOW}} - 1] \times 1,000$ . Modern freshwater cetaceans have  $\delta^{18}\text{O}$  values at least three per mil lower than those of marine cetaceans, consistent with previous findings<sup>8,9</sup>. As body temperature is nearly constant in mammals, the variation in isotope composition of mammalian teeth is a direct reflection of the variation in the isotope composition of the ingested water. The magnitude and direction of the differences of the fossil taxa are consistent with the difference among the modern cetaceans and with sedimentological evidence from the fossil sites in lower Kuldana and Harudi Formations. We analysed oxygen from the dental enamel or dentine (except in *Lipotes*, where only bone was available); each point represents a different individual. Our specimens of *Sotalia* and *Pontoporia* were marine. Phosphate was isolated as  $\text{Ag}_3\text{PO}_4$ , which was thermally decomposed to  $\text{CO}_2$  in the presence of graphite<sup>14</sup>. Resultant  $\text{CO}_2$  was analysed on gas-source mass spectrometers at the universities of Arizona and Michigan. Also summarized are inferred depositional environment and phylogenetic hypothesis for the Eocene taxa. Derived characters supporting the nodes are: 1, sigmoid process, rotated middle ear, pachyosteosclerotic tympanic and incus; 2, peribullar sinuses, enlarged mandibular foramen; 3, supraorbital plate; 4,  $\text{P}^4$  with lingual bulge, lacking protocone.

differences in  $\delta^{18}\text{O}$  values between freshwater and marine cetaceans reflect those of ambient waters, validating our method.

We analysed four of the oldest-known cetaceans. *Pakicetus*, *Nalacetus* and *Ambulocetus* are found in the early to middle Eocene Kuldana Formation of Pakistan. *Pakicetus* and *Nalacetus* are found only in shallow freshwater deposits<sup>11</sup> and probably spent considerable time on land. *Ambulocetus* occurs only in littoral beds<sup>2</sup>. *Indocetus* is known from the Middle Eocene Harudi Formation of western India and occurs only in neritic beds<sup>12</sup>.

The difference in  $\delta^{18}\text{O}$  values between *Pakicetus* and *Nalacetus* on the one hand and *Indocetus* on the other is similar in magnitude and direction to that between modern freshwater and marine cetaceans, suggesting that *Pakicetus* and *Nalacetus* ingested fresh water. This difference is consistent with habitat inferences from sedimentological data. Pakicetids, the geologically oldest whales, were clearly not marine. The  $\delta^{18}\text{O}$  values of *Indocetus* are much higher than those of the lower Kuldana cetaceans and match those of modern marine cetaceans, suggesting that *Indocetus* ingested sea water only. *Indocetus* was thus totally independent of fresh water and the taxon was fully marine.

The  $\delta^{18}\text{O}$  values of *Ambulocetus* are most similar to those of the other Kuldana cetaceans, implying that *Ambulocetus* ingested fresh water. This is surprising, as the taxon is found in unambiguously marine beds high in the Kuldana Formation<sup>2</sup>. Also, *Ambulocetus* has never been found in the freshwater deposits that abound in this part of the Kuldana Formation.

There are two possible explanations. *Ambulocetus*, although it lived in the littoral realm, may have sought out freshwater sources to drink because its osmoregulatory system was unable to handle the excess salt load of its environment. Alternatively, it may have lived in fresh water during the (early) part of its life when its teeth were mineralized, then migrated to the sea later on. This explanation lends credence to the idea that the life history of early cetaceans resembled that of modern pinnipeds<sup>13</sup>.

Our analyses document the origin of seawater ingestion in whales. Our results imply that cetaceans did go through a stage, represented by *Ambulocetus*, in which they lived as adults in marine environments but depended, at least for part of their lives, on a freshwater source. However, by the middle Eocene, the osmoregulatory abilities of neritic

cetaceans had adapted to increased salt loads, allowing them to leave the coast and disperse quickly across the oceans and colonize the globe. Dispersal ability presumably affected the pattern of cetacean macroevolutionary radiations, and may explain their distribution throughout the world's tropical regions only a few million years after their origin on the Tethyan shores of IndoPakistan.

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## Redox regulation of cell signalling

**SIR** — We have been investigating the structural basis of the interaction between nitric oxide (NO) and the *ras* oncogene product p21 to gain insight into how redox signalling is achieved in cells. Our approach is one way to address the more general problem of finding a molecular target for redox-active environmental toxicants and free radicals. We report here that we have identified the site of molecular interaction between NO and p21<sup>ras</sup> that is responsible for the initiation of signal transduction.

In our earlier studies we found that a single S-nitrosylation event on full-length p21<sup>ras</sup> produced enhanced guanine nucleotide exchange<sup>1</sup>. Our present *in vitro* studies used p21<sup>ras</sup> lacking the carboxy-terminal 23 amino acids, as this form of the protein has identical biochemical activity to the wild-type enzyme<sup>2</sup>. To identify the exact site of S-nitrosylation, we cleaved p21<sup>ras</sup> with cyanogen bromide, which yielded three fragments, each containing one cysteine residue. Fragment 1, containing Cys 51, has a relative molecular mass ( $M_r$ ) of 7,203; fragment 2, containing Cys 80, has  $M_r$  4,540; and fragment 3, containing Cys 118, has  $M_r$  6,225.

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We monitored each of the Cys residues for *S*-nitrosylation by subjecting the digested mixture to analysis using electrospray-ionization mass spectrometry (ESI-MS)<sup>3</sup>.

As Fig. 1a shows, CNBr digestion of p21<sup>ras</sup> yields a fragment with  $M_r$  6,223±2, corresponding to fragment 3 (containing Cys 118). Treatment of p21<sup>ras</sup> with NO and subsequent cleavage with CNBr produces a fragment 3 with a new mass clearly indicative of *S*-nitrosylation<sup>3</sup> (an increase of 29±1; Fig. 1a). We did not find fragments 1 and 2.

To confirm that Cys 118 is indeed the site of *S*-nitrosylation, we generated a form of p21<sup>ras</sup> identical to the wild-type enzyme, except that Cys 118 was changed to a Ser residue (referred to as p21<sup>ras</sup>C118S). This modification only changes the sulphur atom of Cys 118 to oxygen, reducing the  $M_r$  of the enzyme by 16, to 18,836. We treated wild-type p21<sup>ras</sup> with NO under conditions which produced approximately 50% *S*-nitrosylation. Analysis by ESI-MS reveals the parent enzyme ( $M_r$  18,852) and a singly *S*-nitrosylated derivative ( $M_r$  18,882; Fig. 1b, blue line). Treatment of p21<sup>ras</sup>C118S with NO under identical conditions gives no *S*-nitrosylated product, but only the parent enzyme (Fig. 1b, red line). These data identify Cys 118 as the molecular target of NO on p21<sup>ras</sup>.

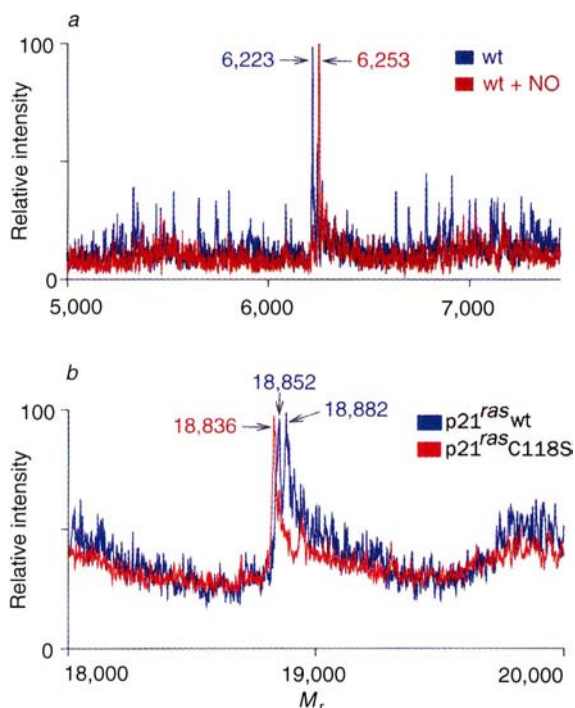


FIG. 1a, ESI-MS analysis of CNBr-digested p21<sup>ras</sup>. Wild-type (wt) p21<sup>ras</sup>, either untreated (blue line) or treated with equimolar NO (red line), was subjected to CNBr digestion and ESI-MS. Representative data from 4 experiments are shown. b, ESI-MS analysis of NO-treated p21<sup>ras</sup> and p21<sup>ras</sup>C118S. Wild-type p21<sup>ras</sup> (blue line) or p21<sup>ras</sup>C118S (red line) was treated with equimolar NO for 10 min before analysis by ESI-MS. Representative data from 3 experiments are shown.

We next examined whether NO could induce nucleotide exchange on GDP-preloaded p21<sup>ras</sup>C118S *in vitro*. NO (25  $\mu$ M) stimulates [ $\gamma$ -<sup>32</sup>P]GTP hydrolysis of GDP-preloaded wild-type p21<sup>ras</sup> 9.34±0.98-fold ( $n=6$ ). In contrast, NO (25  $\mu$ M) has no effect on the exchange rate of p21<sup>ras</sup>C118S (0.98±0.36-fold;  $n=6$ ). Using Jurkat T cells mock-transfected or stably transfected with an expression plasmid encoding a full-length version of p21<sup>ras</sup>C118S (residues 1–189), we examined the ability of NO to activate the kinase activity of mitogen-activated proteins (MAPs). Immunoprecipitation of the MAP kinases ERK1 and ERK2, from wild-type cells treated with the NO-generating compounds *S*-nitroso-*N*-acetylpenicillamine (300  $\mu$ M) or sodium nitroprusside (1 mM), results in enhanced phosphorylation of the ERK substrate (163±8 and 229±12% of control, respectively;  $n=4$ ). In contrast, Jurkat T cells stably expressing p21<sup>ras</sup>C118S (1–189) do not respond to these compounds in this *in vitro* kinase assay (104±6 and 106±3% of control;  $n=4$ ), although the combination of phorbol myristate acetate (100 ng ml<sup>-1</sup>) and the calcium ionophore A23187 (500 ng ml<sup>-1</sup>) stimulates MAP kinase activity in both cell types (221±8 versus 261±9% of control, parental versus transfected;  $n=3$ ).

The crystal structure of p21<sup>ras</sup> is well defined<sup>4,5</sup>; modelling studies show that, of the three Cys residues in our p21<sup>ras</sup> preparation, Cys 118 is most exposed to the solvent (Fig. 2). We calculated the solvent-accessible surface of p21<sup>ras</sup> complexed to GDP. Whereas the Cys 51 and Cys 80 side chains are fairly well shielded from the solvent, Cys 118 is exposed. The fact that residues Cys 80 and Cys 51 are so buried provides a structural explanation for the single *S*-nitrosylation that occurs on p21<sup>ras</sup> on exposure to NO-related species.

These data indicate that Cys 118 is a critical site of

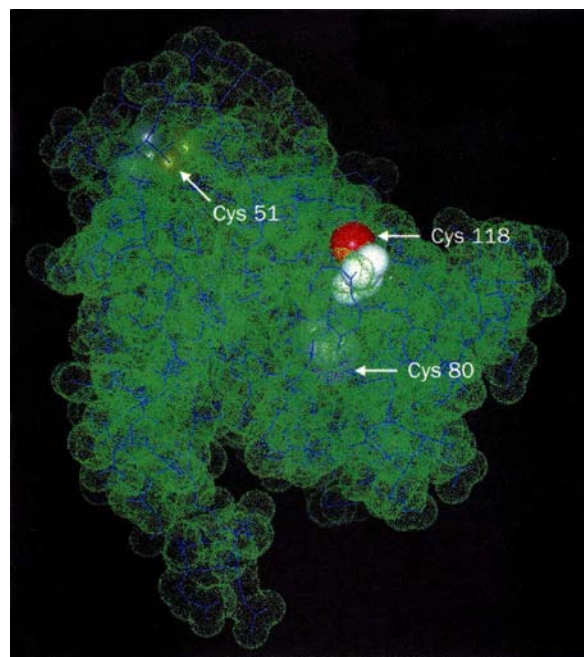


FIG. 2 Surface nature of Cys 118 in the crystal structure of the p21<sup>ras</sup> preparation we studied. Cys side chains (arrowed) are red (Cys 118), purple (Cys 80) and yellow (Cys 51); Cys backbones are white.

redox regulation of p21<sup>ras</sup>, and that *S*-nitrosylation of this residue triggers guanine nucleotide exchange and downstream signalling. Thus, signalling to the nucleus by redox-active species may involve triggering of a molecular switch on p21<sup>ras</sup>.

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# 'Roots' in mixotrophic algae

SIR — Macroalgae are thought to depend on absorption of nutrients from the water column because, unlike vascular plants, they lack root systems with which to exploit nutrient resources in substrata<sup>1</sup>. Here we show that the giant marine coenocyte, *Caulerpa taxifolia*, possesses a 'root system' containing endocellular bacteria which can take up inorganic phosphorus and organic nitrogen from substrata and translocate nutrient products to the photoassimilatory organs. The uptake of carbon, nitrogen and phosphorus from substrata can explain the alga's ability to proliferate in oligotrophic water.

*Caulerpa taxifolia* produces stolons, fronds and gravipositive rhizoids which mimic the organs of a vascular plant. The rhizoids penetrate substrata and attach to mineral surfaces. Chemical action at sites of attachment releases mineral-bound nutrients such as inorganic phosphorus directly to the alga (a in the figure). The rhizoids can also take up dissolved amino acid. Twin-compartment incubation of rhizoids in <sup>14</sup>C-valine-labelled sea water and

attached stolon and fronds in unlabelled sea water demonstrates significant uptake and translocation of <sup>14</sup>C from rhizoids to stolon and fronds (b in the figure). Previous work has shown that NH<sub>4</sub><sup>+</sup> can be absorbed by the rhizoids of *C. cupressoides*<sup>2</sup>. Uptake of nutrients from substrata explains why: neither tissue nutrient content nor photosynthetic rate is correlated with seawater nutrient concentration<sup>3</sup>; seasonal changes in alkaline phosphatase activity are correlated with productivity and not seawater [PO<sub>4</sub><sup>3-</sup>] (ref. 3), and elevation of seawater [PO<sub>4</sub><sup>3-</sup>] does not inhibit alkaline phosphatase activity<sup>3</sup>, as occurs in other marine algae<sup>4</sup>. These data indicate that nutrient uptake from the water column is subordinate to uptake from substrata.

Mixed bacteria coat the outer surfaces of the rhizoids, whereas large numbers of bacterial rods (10<sup>4</sup>–10<sup>5</sup> mm<sup>-3</sup>) exist in the cytoplasm. We isolated several different bacteria from cytoplasmic fluids aspirated from the rhizoids and cultured two Gram-negative, polarly flagellated, aerobic bacteria, with differing urease activity (A and

B). The 16S ribosomal RNA sequences of these organisms<sup>5</sup> place bacterium A, a halophile, close to unnamed species of *Rhodopseudomonas* in the *Agrobacterium-Rhizobium* group of branch-α proteobacteria, and bacterium B in the fluorescent *Pseudomonas* group of branch-γ proteobacteria<sup>6</sup>. Bacterium A carries the *nifH* gene, which encodes nitrogenase production<sup>7</sup>. A *Rhodopseudomonas*-like bacterium with the potential to fix N<sub>2</sub> in the rhizoids of an alga has not been reported.

*Caulerpa taxifolia* has recently proliferated in the northwest Mediterranean on substrata that are heavily enriched in organic matter, largely anoxic, perfused with hydrogen sulphide and contaminated with precipitated inorganic phosphorus. Oxygen deficits in rhizoids rooted in anoxic substrata could support N<sub>2</sub> fixation<sup>8,9</sup>, thus accelerating decomposition of high C:N macrophyte vegetation<sup>8</sup> and hence nutrient supply to the alga. Removal of sulphide by a *Rhodopseudomonas*-like bacterium<sup>10</sup> could give the alga a further advantage by protecting its rhizoids. Catabolism of organic substrates by endocellular and surface-borne heterotrophs could supply CO<sub>2</sub> for photosynthesis and partially explain the highly negative δ<sup>13</sup>C tissue values (<–30‰) reported in other *Caulerpa* spp.<sup>11</sup>.

Further research must establish how the alga and bacteria interact, whether N<sub>2</sub> fixation occurs, and the chemical mechanism by which inorganic phosphorus is acquired by the rhizoids. Our results show that the rhizoids of *C. taxifolia* function as 'roots' and can derive organic carbon and nitrogen, and inorganic phosphorus, directly from substrata. Rhizoid uptake of nutrients provides a physiological explanation for the success of siphonaceous green algae in oligotrophic tropical waters and the recent prolific development of *C. taxifolia* on eutrophic substrata in the northwest Mediterranean.

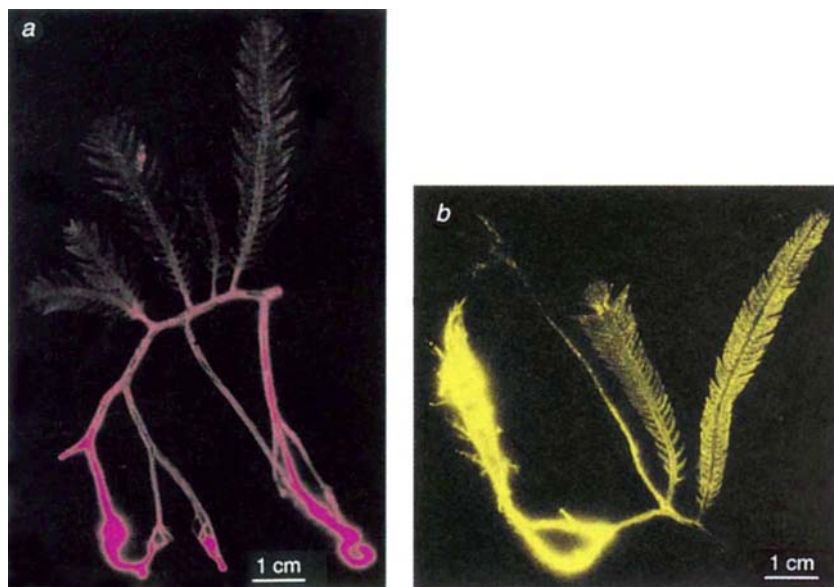
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Autoradiographs demonstrating uptake of inorganic and organic nutrients by the rhizoids of *C. taxifolia* and their subsequent translocation to the photoassimilatory stolons and fronds. a, We grew test and control samples of *C. taxifolia* adjacent to one another for 7 days in flow-through aquaria containing a 1-cm-deep, aerated bed of coarse CaCO<sub>3</sub> sand (particle size 3–5 mm) labelled with a surface precipitate of <sup>33</sup>P-apatite (8.9 kBq cm<sup>-3</sup> substrate). We prepared the precipitate by agitating the sand grains for 6 h in a minimal volume of sea water containing H<sub>3</sub><sup>33</sup>PO<sub>4</sub><sup>3-</sup>; we removed non-precipitated <sup>33</sup>P by flushing (6 h). We grew rhizoids of the test samples for a 7-day period until they had penetrated and attached to the substratum; we prevented rhizoids of control samples from making contact with the substratum. We then removed samples, rinsed them in fresh sea water, freed them of adhering sand grains, and pressed, dried and autoradiographed them. Test samples showed strong uptake of <sup>33</sup>P by the rhizoids and translocation of <sup>33</sup>P to the photoassimilatory parts, whereas control samples failed to produce an image on the autoradiographic film plate. b, We incubated rhizoids in sea water containing <sup>14</sup>C-valine (11.1 kBq ml<sup>-1</sup>) in one half of a slotted, twin-compartment, incubation vessel that allowed adjoining stolon and fronds to be incubated simultaneously in unlabelled sea water. Communication between the two incubation compartments (other than through the algal siphon) was prevented by sealing the junction between them with Vaseline, and gaseous exchange of respired <sup>14</sup>CO<sub>2</sub> was prevented by capping the rhizoid compartment. Significant uptake of <sup>14</sup>C-valine by the rhizoids, and translocation of <sup>14</sup>C to the stolon and fronds, occurred after 20 h.

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# Science and its discontents

A. J. Meadows

**Science and the Retreat from Reason.** By John Gillot and Manjit Kumar. *Merlin*: 1995. Pp. 287. £18.95 (hbk); £10.95 (pbk).

**Newton's Sleep: Two Cultures and Two Kingdoms.** By Raymond Tallis. *St Martin's/Macmillan*: 1995. Pp. 260. \$49.95, £42.50 (hbk); £15.99 (pbk).

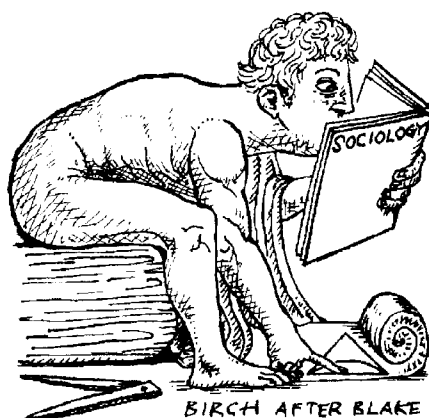
**Misunderstanding Science? The Public Reconstruction of Science and Technology.** Edited by Alan Irwin and Brian Wynne. *Cambridge University Press*: 1996. Pp. 232. £35, \$59.95.

How should we think about scientific knowledge? Although these three books deal with different topics, this question is the common theme running through them. John Gillot and Manjit Kumar are particularly concerned with the idea of progress in science. Raymond Tallis contrasts the two worlds of science and art. Alan Irwin and Brian Wynne present a series of case studies on the public understanding of science. But in each book the question arises how science should be seen in the context of other activities, more especially those studied by way of the humanities and social sciences.

In an extreme form, the question might be posed this way: is scientific knowledge a mountain or part of a plateau? If it can be equated to a mountain, its appearance will change with one's viewpoint; but it will always be a distinctive feature in the intellectual landscape. If science is part of a plateau, although it may occupy a recognizable part of the domain, it will merge seamlessly into surrounding modes of knowing. Tallis sums up the latter image in the following way: "Science has no privileged access to the truth about the natural world: its special authority is socially constructed". In his preface, he explains that his medical training leads him to disagree totally with this assertion, adding: "If science were merely the art of persuasion... then it is impossible to see how science could ever have been effective". This contrasts with Irwin and Wynne's introduction: "In this book, we will draw upon the last two decades of research within the sociology of scientific knowledge which has convincingly demonstrated the *socially negotiated* nature of science" (their emphasis).

To anyone with a passing awareness of the latest trends in the sociology of science, this is a familiar battleground. But the debate contains many ramifications. For example, when William Blake decried "Newton's sleep", he accepted science's separate existence but believed that its adherents were blinkered. This represents a different sort of attack on scientific thinking, most famously carried forward in the 'two cultures' debate 35 years ago (to which Tallis devotes a chapter), but not altogether absent today. Thus queries have been

raised about the symbolism implied by the statue of Newton, based on Blake's vision, that now fronts the new British Library building, just a stone's throw from *Nature's* London office. (Any subtle support for the humanities that the statue may represent has, unfortunately, been drowned by the library's appearance over the past few



years. It has been wrapped like a birthday present awaiting some long-delayed celebration — perhaps a far-from-subtle comment on the position of knowledge of all kinds today.)

Gillott and Kumar explore another aspect of the dichotomy between science and society: the extent to which the concept of progress in science needs to be kept distinct from the same concept applied to society. They argue that the Enlightenment idea of progress included the assumption that humanity could mould nature. This contrasts with the prevailing preference for non-intervention in nature. The changing views of society and its relationship to the world about us have led to a denigration of the idea of scientific progress. This has also manifested itself in a gradual retreat from a belief in reason. The two come together in asserting the primacy of society over science. The authors point out, in passing, that the link was made long ago by Hitler (who certainly thought that the Age of Reason was over): "Science is a social phenomenon, and... is limited by the usefulness or harm it causes".

The scientists seem firm in drawing an ultimate distinction between science and

society. What, then, of the sociologists? "Implicit", say Irwin and Wynne, "in our collection [of essays] is that only a properly sociological approach to contemporary science can give us a real insight into the issues of 'public understanding'." Clearly, the scientists do not find the sociologists' assumptions helpful, and vice versa. Can one progress further?

Two points are obvious. We all see through the particular methodological spectacles that we have learnt to wear. Sociological methods applied to science tend to produce a picture of science that makes it look strangely like sociology. (The same has been true, in reverse, of some past attempts to impose scientific methodology on sociology.) Equally, science is not an entirely social activity. It has, for example, an important cognitive element. From both viewpoints, sociology can be expected to produce only a partial view of science. The question, therefore, is what are the limitations of a sociological dissection of science, and so of the helpfulness of sociological investigations?

It is exasperating that, although the individual authors in Irwin and Wynne's book are aware of this basic problem, they say little about it. For example, it is wryly observed, at one point, that sociologists of science have a tendency to regard 'science' as problematic, but not 'society'. The ethnographic viewpoint that pervades the contributions in this volume might well have been examined usefully in this context. Again, one aspect of cognition is discussed briefly — under the heading "mental models" — but mainly in order to criticize the approach for what it neglects.

There is nothing new in this picture of two intellectual groups ploughing separate furrows. Philosophers of science have been explaining how science works, and scientists have been ignoring them, for many years past. But understanding how the interfaces work between science and its various publics is becoming increasingly important to scientists. This may be one explanation of the scientific backlash against what are seen as the relativistic leanings of some commentaries on science. It will be a pity if this gap between the differing viewpoints cannot be bridged. The case studies in Irwin and Wynne's volume, for example, contain several insights that scientists would find stimulating. It is said that new developments in university teaching are encouraging a 'mix-and-match' philosophy of learning. Maybe that is actually the way for scientists and sociologists to learn from each other in the immediate future. □

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## The academic sphere

David E. H. Jones

**Science of Fullerenes and Carbon Nanotubes.** By M. S. Dresselhaus, G. Dresselhaus and P. C. Eklund. *Academic*: 1996. Pp. 965. \$130, £95.

A HIMALAYAN expedition once set out to track down the elusive Yeti. Reporters were waiting at the base camp when the team returned. "Did you find it?" they asked excitedly. "Have you captured a Yeti?" "Well, no", replied the leader; "But

physicist's part of the picture — the symmetry properties of  $C_{60}$  and its derivatives, their crystal structures and mass-spectral characteristics, their vibrational, electronic, transport, magnetic, optical and surface-scattering properties — in clear detail and with confidence.

By contrast, the chemist's side of the picture is sketched much more tentatively. Here is an actual chemical substance: a new allotrope of carbon, perhaps the first new allotrope of any element to have been discovered this century. What does it look like? How do you handle it? What can you do with it? Such concrete questions arise most naturally in the chapters covering the preparation of  $C_{60}$  and its chemical properties, and are not well answered.

The topics are carefully referenced, but the text itself lacks chemical detail. It describes many new compounds with a  $C_{60}$  moiety in the molecule, but is content to give their proposed structures. What effect does this massive substituent have on the characteristic properties of these compounds? How stable are they? What new tricks do they get up to? Do not enquire within.

The melting point of  $C_{60}$  provides another example. In the table of properties, it is given as 1,180 °C. A sketched phase diagram shows its triple point at about 1,800 °C and 13 bar. The caption warns that this is an extrapolation and that the existence of a liquid phase remains to be proven. Now  $C_{60}$  is stable at

well above 2,000 °C, as shown by its synthesis in a carbon arc. It ought to melt without decomposition, although its liquid range might be very narrow. So why has nobody subjected  $C_{60}$  to 1,800 °C and 13 bar? Again, the authors are silent, apart from providing references. A short search from these starting points turned up one paper saying how interesting it would be to melt  $C_{60}$ , three ingenious but divergent calculations about its possible melting point, but no actual attempts to melt it. Clearly the authors are not alone in treating  $C_{60}$  research as largely a theoretician's playground. If the book were a novel, I'd have to say that the central character never comes alive.

This is a pity, because  $C_{60}$  and its relatives have caught the collective chemical imagination in a way few other molecules have done. It may well be the most intensely researched single molecule in modern chemistry. And yet its study remains oddly introverted. On the evidence of this book, fullerene research is entirely about fullerenes. It is not about the impact of fullerenes on other aspects of science or technology, because (as yet) there is almost no such impact. Even the early brave hope that  $C_{60}$  would turn out

to be the preferred form of interstellar carbon has not been borne out. The final chapter scrapes together a few potential applications — photoresists, lubricants, photoconducting surfaces and so on — but nothing to get excited about. By and large, a scientist or technologist outside the field still has no professional reason to pay any attention to it.

This seems almost incredible, and it surely cannot last. The fullerenes must have wider implications than their mere novelty. I am reminded of the curious limbo in which the newly invented laser found itself in the early 1960s. It was universally recognized as an exciting device, a splendid piece of new physics, but nobody had much idea of what it might be good for. It took at least a decade for the laser to 'grow up' and start to make its mark on physics, chemistry and technology. I remain optimistic that the fullerenes will follow the same sort of trajectory. Watch this space! □

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■ *Perfect Symmetry: The Accidental Discovery of Buckminsterfullerene* by Jim Baggott is now out in paperback (Oxford University Press, £9.99). See *Nature* **372**, 514 (1994).

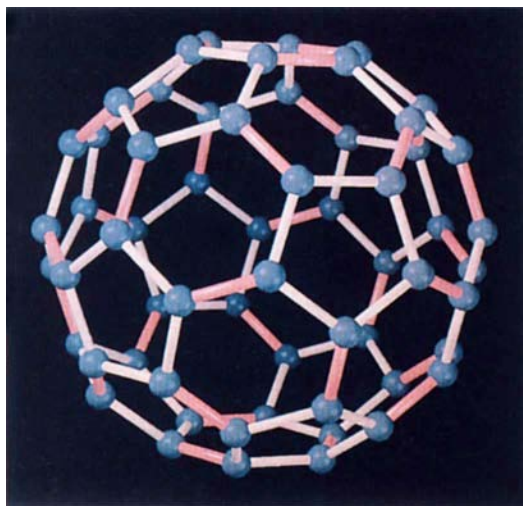
## Climate reversal

Stephen H. Schneider

**Global Warming: The Truth Behind the Myth.** By Michael L. Parsons. *Plenum*: 1995. Pp. 271. \$27.95.

MICHAEL Parsons claims the scientific and ethical high ground of 'truth' in this crusade against "professional environmentalists and others whose jobs depend upon the continuing environmental crises". He also attacks the "many scientists [who], for completely different reasons, fuel a fire of hysteria with 'data-free speculation'" because "career pressures have become very strong in contemporary science". "Investigating fraud in science", he says, "tells us a lot about these pressures".

Parson thus implies that it is dishonesty that has motivated 'consensus' scientists, such as those on the Intergovernmental Panel on Climate Change (IPCC), to express serious and growing concern about global warming. He claims, for example, that "some scientists and many environmental activists warn that increases in anthropogenic greenhouse gases might cause a runaway global warming effect". But I am not aware of any scientist or environmental activist who has ever used the phrase 'runaway global warming' in the context of Earth (the jargon is usually reserved for the oven-like super-green-



Supercomputer simulation of the atomic structure of a  $C_{60}$  molecule.

we do have spectroscopic evidence for its existence".

Buckminsterfullerene, the famous spherical graphite molecule, still seems haunted by its spectroscopic past. Originally identified in 1985 as a remarkably intense peak corresponding to  $C_{60}$  in a mass spectrum, it became available in gram quantities in 1990 by direct synthesis from graphite in a low-pressure carbon arc. And yet, in the pages of this book, it still exists largely as a trace on the screens of an ever wider suite of instrumentation, and the subject of arcane calculations seeking to explain that trace.

This approach is understandable. The novel form of the molecule, its high symmetry and intriguing bonding, pose a delightful set of tricky challenges to the theorist. Even better, the challenge extends to many related compounds — the isomeric and higher fullerenes, the doped, metallated and substituted fullerenes, and carbon nanotubes. Furthermore, the resulting predictions can usually be squared quite well with the spectroscopic findings. This book claims to be the first comprehensive guide to the whole field of fullerenes, and is copiously referenced up to 1995. It presents the



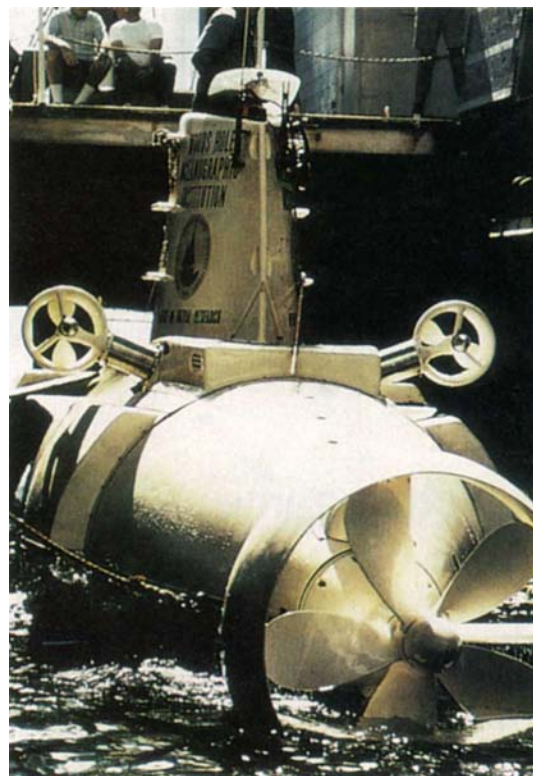
house effect on Venus); and, needless to say, these dissembling activists and scientist-hysterics go unnamed and uncited in Parsons's text. Here, it seems, is yet another anti-environmental backlash book, joining those of Dixy Lee Ray, Rush Limbaugh, Greg Easterbrook and others of this emerging genre.

The first test of a myth-versus-truth book is how well it handles the science. Sadly, the most I can do in the space available here is to give a flavour of the scientific acumen of the author. Parsons speaks admiringly of the research of the climate contrarian Sherwood Idso, describing it as "using the empirical approach, different from that of the modelers who worked with numerical theory". What Parsons misses is that Idso, even if unwittingly, also worked with 'numerical theory' but overlooked well-known categories of energy flows and storage factors. Nor does he mention that the US National Academy of Sciences (NAS) took the unusual step of devoting an entire section in its *Second Assessment of Carbon Dioxide and Climate* in 1982 to debunking the scientific errors made by greenhouse dissenters through these kinds of miscalculations.

Parsons flatly states: "You probably have not read about model validation in any of the popular literature. The omission of discussion about validation stems from the fact that current computer climate models cannot be validated by these techniques. That is, they do not accurately predict known weather on a global basis." Here he is simply confusing the nature of weather forecasting, an 'initial value problem', in which an initial condition is integrated forward until its information is lost in the chaotic dynamics of the atmosphere some weeks later, and a 'boundary value problem', in which some external 'forcing' factor such as solar energy input, volcanic dust veils or carbon dioxide is changed and the subsequent climatic statistics are perturbed. These differences are well captured by most computer models — a validation point missed by Parsons, but not by IPCC or NAS scientists. Instead, Parsons focuses on a technique — sequence of climate anomalies — that cannot in principle be a definitive validation test because of the chaotic dynamics of the atmosphere.

And when he tries to explain chaotic dynamics, he is completely confused: "Linear equations are solvable; if you know the input data, you can calculate a specific solution. Nonlinear systems cannot be so simply treated, they are not solvable. One of the most important traits of a nonlinear system is its sensitivity to initial conditions." A parabola is a nonlinear equation, and so is the energy conservation law for the atmosphere. Their complexity is, of course, radically different, but it is not generally true that nonlinear systems are "not solvable", nor are nonlinear

**ROBERT Ballard, the man who led the expedition that discovered the wreck of the *Titanic* in 1985, climbs aboard *Alvin*, the US Navy's three-man research submersible. The picture is taken from *Explorations* by Ballard and the writer Malcolm McConnell, now published in paperback by Hyperion at \$14.95. Reviewing the hardback edition last year, Charles H. Langmuir wrote: "Ballard reveals himself as a superb salesman, capable of delivering a glamorous view of sea-going exploration as mythic accomplishment.... Those interested in deeper questions of discovery and the nature of man and science will need to look elsewhere".**



systems necessarily inscrutable with regard to their dependence on initial conditions. Certain types of nonlinear systems with chaotic behaviour may well be inordinately sensitive to initial conditions. But that does not mean that even these difficult nonlinear systems are unpredictable with regard to certain changes in their boundary conditions, such as sunlight, which Parsons himself expects to be an important driver of climate change (or volcanic eruptions, or carbon dioxide for that matter).

Parsons also says that the predictions of different general circulation models do not correlate with each other. In fact, such correlation among these models and climate data was the basis for the recent IPCC assertion that, while it recognized many remaining uncertainties, "the balance of evidence suggests a discernible influence of human activities on climate".

To me, the deeper issue here is why a usually responsible publisher such as Plenum is putting out this kind of book. How did it get through the normal scientific review process? How did major misstatements and personal values masquerading as science get through the quality-control filters? I can understand how this happens with books published or paid for by anti-environmental, ideological organizations such as the Cato Institute, whose authors repeatedly distort global climate-change science, apparently fearing that corrective policy action may involve national and international rules that might

hurt their interests.

Parsons says, as if it were a fact or a scientific conclusion, that "governmental action should be taken only when scientific theories are substantiated more concretely than has been done in the case of global warming". How much objective certainty does one need before undertaking a medical procedure, making a business investment, buying a defence system or even in some cases going to war? If Parsons had said that his personal value judgement was to be environmentally risk-prone and economically risk-averse — preferring to risk environmental change rather than risk investments to slow down that change — fine. But he does not differentiate issues of facts from issues of values, nor does he present a sophisticated policy analysis, or a good cost-benefit framework, or a discussion of the relative merits of the precautionary principle compared with a discounted economic efficiency scheme. All this would be manifold in any balanced, state-of-the-art policy analysis of this issue.

I do not support elite censorship or the suppression of heterodox points of view. But I do expect technical books to be subjected to a high standard of technical review. Although Parsons may be legitimately angry about overstatements from some environmental extremists — and indeed there are such statements — he holds no equal disdain for the scientifically absurd statements by the likes of Dixy Lee Ray and for Sherwood Idso's flawed climate theories. Nor, for that mat-

ter, does he doubt the often questionable and certainly contrarian pronouncements from S. Fred Singer, who wrote the preface to the book and is quoted favourably throughout. My hope is that reputable publishers will solicit independent opinions before embracing self-proclaimed 'truth' books and that readers will learn to be wary of unbalanced books purporting to tell balanced truths, which for the most part turn out to be the real myths. □

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## Prediction game

Andrew Watson

**Living Dangerously: The Earth, Its Resources, and the Environment.** By Heinrich D. Holland and Ulrich Petersen. Princeton University Press: 1996. Pp. 490. \$49.95, £30 (pbk).

FOURTEEN children are named at the beginning of this book — the grandchildren of the authors, to whom they dedicate it. And reading the text is in many ways like being taken on a tour of the planet's resources by a kindly grandfather, full of the wisdom and caution that (sometimes, at least) comes with age. The book covers a rich, almost bewildering range of subjects, which might be expected to make the head spin, but it has been carefully written (and benchmarked, the authors say, extensively on students) so that it manages to flow seamlessly across the disciplines from physics to geology, to economics, to chemistry and back again. In case one's attention starts to flag, there are grandfatherly asides from time to time — newspaper cartoons, quotations from the classics, reference to modern classics such as "Star Trek" and the like. Overall, it is a *tour de force* — a weighty and informative textbook that is always user-friendly and enjoyable to read.

The authors provide a nonspecialist, undergraduate-level account of the status of the Earth's resources and the problems we and our children will face in the next hundred years. Little basic knowledge of the natural sciences is assumed, so there are digressions on, for instance, atomic structure, crystal lattices and basic chemistry slotted in as necessary among a comprehensive coverage of most aspects of 'Earth system' science. As might be expected, there are sections on today's great environmental threats and debates, including global warming, stratospheric ozone depletion and deforestation. But



THE African boomslang snake *Dispholidus typus*. Because its unusually long fangs are further forward in the mouth than other back-fanged snakes, it can cause a very dangerous bite. From *Nightmares of Nature: The Truth Behind the World's Most Dangerous Animals* by Richard Matthews, which accompanies a recent BBC television series. HarperCollins, \$24, £14.99.

there is also plenty of space given to less global but more immediate problems, for example flood prevention and control, or present and future water supply and food provision for the developing world. We are challenged to ask whether it is more important to be concerned with global warming, which may in the future affect billions of people, but which has probably not so far been responsible for a single death, or with the provision of clean water in developing countries, for want of which it is estimated that 5 million children die each year.

People's attitude to the future is determined largely by their own temperament. The authors declare themselves to be "cautiously optimistic". Although they are careful to avoid the excesses of the slash-and-burn exploitation, they firmly reject prophecies of ecocatastrophe, seeing no inevitable crises over food, resources or energy. If we can have just a little foresight, they seem to say, our grandchildren could inherit a world that is really quite pleasant, neither wholly polluted nor exhausted of all natural resources.

They make a good argument, and they almost convinced me, usually a confirmed pessimist. But the annoying thing about the future is that it is so unpredictable. Unforeseen and unforeseeable events such as new technologies, epidemics or wars will probably be what shapes it. This is an enjoyable and

admirable book, but although the authors show us what *might* happen, in the end a crystal ball would be almost as useful in predicting what actually will happen in the next century. □

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### New Journals

This year, *Nature's* annual New Journals review supplement will appear in the issue of 5 September. Publishers and learned societies are invited to submit journals for review as well as details of any eligible electronic journals. Journals that first appeared during or after June 1994 and issued at least four separate numbers by the end of May 1996 will be considered. Frequency of publication must be at least three times a year and the main language used must be English. Deadline for submission is 14 June.

When submitting journals for review, please send at least four different issues (the first, the most recent and any two others) of each title, together with full details of subscription rates. For further information please contact Peter Tallack, *Nature*, Macmillan Magazines Ltd, Porters South, Crinan Street, London N1 9XW, UK. Tel: +44 (0)171 843 4567; e-mail p.tallack@nature.com.

# Two distinct mechanisms for long-range patterning by Decapentaplegic in the *Drosophila* wing

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**Secreted signalling molecules provide cells with positional information that organizes long-range pattern during the development of multicellular animals. Evidence is presented that localized expression of Decapentaplegic instructs cells about their position along the anterior–posterior axis of the *Drosophila* wing in two distinct ways. One mechanism is based on the local concentration of the secreted protein; the other is based on the ability of the cells to retain an instruction received at an earlier time when their progenitors were in close proximity to the signal. Both mechanisms are involved in axis formation.**

The possibility that secreted signalling molecules act as morphogens has often been invoked as a mechanism to explain long-range patterning in multicellular fields (reviewed in refs 1–4). Part of the appeal of this mechanism lies in its simplicity. It is easy to understand how a concentration gradient can be established by diffusion of a signalling molecule from a localized source, and how cells might read out their fate by differential expression of target genes in response to the local concentration of the signal. It has been demonstrated in *Xenopus* that activin, a secreted signalling molecule of the TGF- $\beta$  family, can specify distinct cell fates in a manner that appears to correlate with its local concentration<sup>5–8</sup>. Similarly, the *Drosophila* TGF- $\beta$  family member Decapentaplegic (Dpp) is thought to function as a morphogen in dorsal–ventral (D/V) axis formation in the early embryo<sup>9</sup>.

Dpp has also been proposed to function as a morphogen along the anterior–posterior (A/P) axis of the *Drosophila* wing<sup>10,11</sup>. Expression of Dpp in a stripe of anterior cells adjacent to the A/P compartment boundary depends on local induction by the signalling molecule Hedgehog, expressed by posterior cells<sup>12,13</sup>. Localized expression of Dpp serves as a relay signal to control growth and specify cell fate along the A/P axis of the limbs<sup>10,11,14–16</sup>. To do this, Dpp must specify cell fates in a symmetric manner in both anterior and posterior compartments, that is, Dpp expression must further subdivide these compartments by defining new domains of gene expression centred on its domain of expression along the A/P boundary. Here we identify two target genes, *spalt* and *optomotor-blind* (*omb*), through which Dpp acts to specify cell fates along the A/P axis of the wing. We analyse whether the activation of these target genes can be correlated with the local concentration of Dpp, as would be required if Dpp is to be considered as a morphogen for A/P axis formation. We present evidence for two different mechanisms by which localized expression of Dpp can organize long-range pattern, only one of which is based on the local concentration of Dpp.

## dpp-dependent target genes

The *spalt* and *omb* genes have been identified as candidates to mediate the activity of Dpp and A/P patterning by their expression patterns in the wing disc. The *spalt* gene encodes a zinc-finger protein<sup>17</sup> expressed in a broad stripe centred on the stripe of Dpp expression (Fig. 1a). *omb* encodes a member of the T-box family of

transcription factors<sup>18</sup> and is expressed in a broader domain, also centred on the Dpp stripe (Fig. 1d; see also ref. 19). Double labelling for Spalt and *omb-lacZ* show that the Spalt domain is nested within the *omb* domain (Fig. 3a). Two lines of evidence show that the spatial domains of Spalt and *omb* expression are defined by Dpp activity. First, Spalt and *omb* are not expressed in wing discs mutant for *dpp* (Fig. 1b, e). Note that Spalt has additional domains of expression in the wing hinge and notum that are not related to the Dpp stripe and are not regulated by *dpp* (Fig. 1a, b). Second, ectopic expression of Dpp directs spatially inappropriate expression of Spalt and *omb* (Fig. 1c, f). A *vestigial-Gal4* driver line was used to direct expression of a *UAS-dpp* transgene along the D/V boundary of the wing (perpendicular to the endogenous domain along the A/P boundary). Under these conditions, Spalt expression expands to fill the wing pouch (Fig. 1c); *omb* expression extends farther, including the wing hinge (Fig. 1f).

To relate the domain of *omb* expression more precisely to the structure of the wing, an *omb-Gal4* driver line was used to direct expression of the *UAS-yellow+* and *UAS-GFP* (green fluorescent protein) reporter genes (Fig. 2a, b). The broad domain of *omb* expression in the wing imaginal disc corresponds to a wedge of expression that extends anteriorly from midway between veins 1 and 2, across the compartment boundary, to midway between veins 4 and 5 in the posterior compartment. Reducing *omb* activity leads to deletion of a central domain of the wing (Fig. 2c). In a stronger mutant, a more extensive domain is deleted (Fig. 2f). Analysis of clones of cells mutant for the same strong *omb* allele show that mutant cells are tolerated at the extreme anterior and posterior edges of the wing, where *omb* is not expressed (Fig. 2d, e), but the same clones cause deletion of mutant tissue from the centre of the disc, where *omb* is expressed (see also ref. 19). Thus the region in which *omb* activity is required correlates well with its domain of expression. A slightly narrower central domain of the wing depends on the activity of Spalt and its closely related homologue *spalt-related*<sup>20</sup>. These observations indicate that the nested domains of *omb* and *spalt/spalt-related* gene expression are required for the proper development of the stripes of wing tissue in which the genes are expressed.

## Effect of Dpp concentration on stripe width

To determine whether the local concentration of Dpp directly



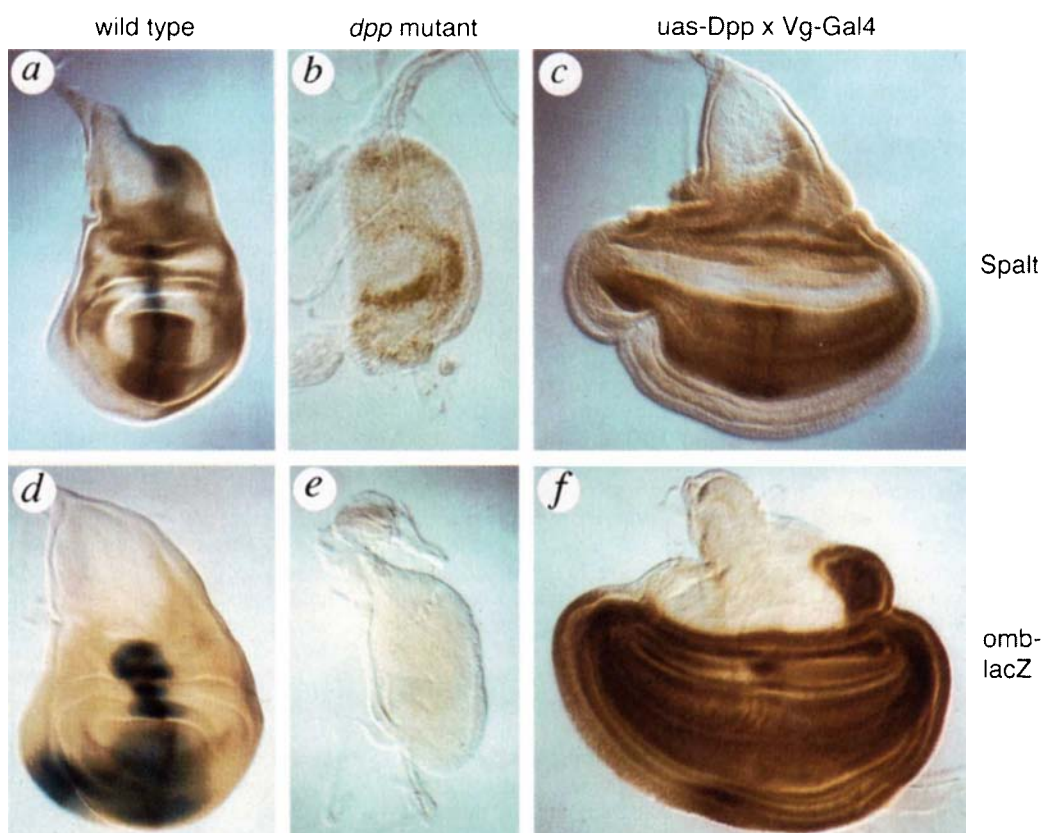
defines the width of the Spalt and *omb* stripes, we investigated whether overexpression of Dpp in its normal spatial domain would cause the Spalt and *omb* domains to expand to form broader stripes along the A/P axis of the wing. A *dpp-Gal4* driver line was used to direct *UAS-dpp* expression in cells already expressing the endogenous *dpp* gene. By using an antibody to Dpp,<sup>21</sup> we verified that Dpp is expressed at a higher level and that the secreted protein is seen in a broader stripe than in wild-type discs (data not shown). Overexpression of Dpp in its normal domain causes a dramatic expansion of the wing along the A/P axis (Fig. 3b, c). The width of the Spalt domain is expanded relative to the Dpp stripe, as would be expected if secreted Dpp directly defines the domain of Spalt expression (compare Figs 3b and 1a). A *UAS-lacZ* reporter gene was included to mark the cells in which Dpp was overexpressed by the *Gal4* driver. Surprisingly, overexpressing Dpp does not have a corresponding effect on the spatial domain of *omb-lacZ* expression. The *omb* domain does not appear to be substantially broadened, and double labelling shows that the *omb* and Spalt domains are equally broad under these conditions (Fig. 3c). Note that Fig. 1 shows that there is no inherent limit on the extent to which Spalt and *omb* expression can be broadened by Dpp expression across the wing pouch. Given that expression of both target genes depends on Dpp activity, and that *omb* is normally expressed at a greater distance from the source of the Dpp signal, the morphogen model predicts that *omb* should be activated at a lower concentration of Dpp than is Spalt. If Spalt and *omb* both depended on the local concentration of Dpp, the *omb* domain should be broader than the Spalt domain when Dpp

is overexpressed from a localized source; however, this does not appear to be the case.

If the extent of the Spalt domain is defined by the local concentration of Dpp, we would expect that all cells expressing Spalt must be able to sense the Dpp signal directly. To address this point, we have made clones of cells mutant for a component of the Dpp signal transduction pathway. The *Mothers against Dpp* (*Mad*) gene was identified by genetic interaction with *dpp*<sup>22,23</sup>. *Mad* has recently been shown to act downstream of the Dpp receptor *thick veins* (*tkv*), suggesting that *Mad* is required for transduction of the Dpp signal<sup>24</sup>. Cells with reduced *Mad* activity fail to express Spalt (Fig. 4a); even clones that overlap only the edge of the domain remove Spalt expression in the mutant cells (arrows). This observation suggests that cells must be able to transduce the Dpp signal to express Spalt. The domain of Spalt expression therefore provides a minimum estimate for the range of the Dpp signal.

To compare the responses of Spalt and *omb* to a localized source of ectopic Dpp, we examined the ability of clones of Dpp-expressing cells to induce Spalt and *omb* expression. As expected, Spalt is expressed at a considerable distance away from the ectopic source of Dpp (Fig. 4c), whereas *omb* expression is more restricted to the region near the clone (Fig. 4d). Although *omb* can be turned on non-autonomously by Dpp, these observations suggest that the effective range of Dpp in activating *omb* is less than it is for Spalt. Given that the extent of the Spalt domain appears to be defined directly by the range of the Dpp signal, these results suggest that this is unlikely to be the case for *omb*.

FIG. 1 The spatial domains of Spalt and *omb* expression are regulated by *dpp*. a, Double labelling to visualize Spalt (brown) and *dpp-lacZ* expression (dark blue). The Spalt domain is centred on the stripe of *dpp* expression. Note that Spalt is also expressed at the periphery of the disc in the hinge region and in the notum, in patterns unrelated to the domain of *dpp* expression. b, Spalt expression in a *dpp* mutant disc (genotype *dpp<sup>Δ12</sup>/dpp<sup>Δ14</sup>*). The central stripe of Spalt expression is missing, but expression in the notum and hinge remain. c, Ectopic expression of Dpp along the D/V compartment boundary using a *vestigial-Gal4* driver causes ectopic expression of Spalt throughout the wing pouch (genotype *Vg-Gal4/+; UAS-Dpp/+*). d, Double labelling to visualize *omb* expression using a *lacZ* reporter gene (dark blue) and the position of the A/P compartment boundary by antibody to the Patched protein (brown). Patched is expressed throughout the anterior compartment at low levels, but is increased in expression in a stripe of cells adjacent to the compartment boundary<sup>31</sup>. This is the same domain in which *dpp* is expressed. e, There is no *omb-lacZ* expression in a *dpp<sup>Δ12</sup>/dpp<sup>Δ14</sup>* mutant disc. f, Ectopic expression of Dpp (genotype *Vg-Gal4/+; UAS-Dpp/+*) causes ectopic expression of *omb-lacZ* throughout the wing pouch and in the surrounding wing hinge region. The difference in the ectopic domains of Spalt and *omb* is consistent with the difference of the proximal limits of expression of the endogenous genes. Note the small bulge of *omb*-expressing cells that is



associated with an unusual fold in the disc. This appears to be a small duplication of hinge structures in the posterior compartment.

**METHODS.** Mouse antibody to Patched was provided by W. Norris and P. Ingham. Rabbit antibody to Spalt is described in ref. 17, *UAS-dpp* in ref. 32, *Vestigial-Gal4* in ref. 33. *ln(2L) dpp<sup>Δ12</sup>* and *Df(2L) dpp<sup>Δ14</sup>* are mutants that remove most or all of the imaginal disc function of Dpp<sup>34</sup>.

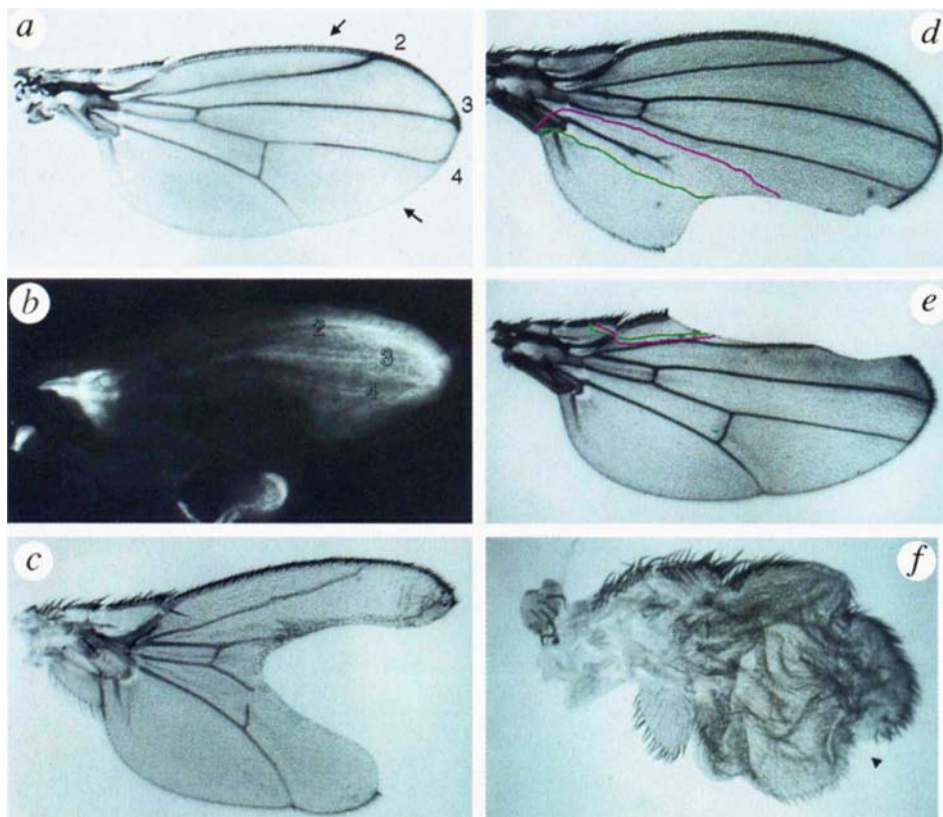
## Non-autonomous signalling by Dpp

Is non-autonomous signalling by Dpp important for long-range patterning of the A/P axis? Because the two target genes behave differently in response to Dpp expression, we asked to what extent the production of nested expression pattern in the wing disc depends on the long-range activity of secreted Dpp. One way to assess the requirement for Dpp secretion in the generation of this pattern is to mimic the effects of Dpp signalling by cell-autonomous activation of the Dpp signal transduction pathway (see ref.

25 for a similar analysis of the Wingless signalling pathway).

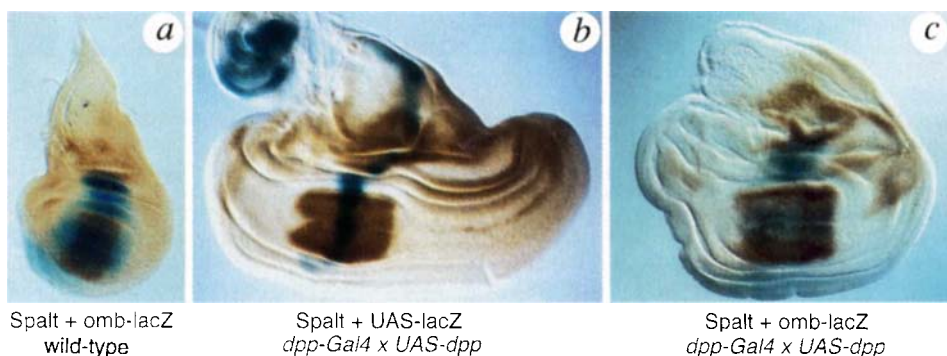
Previous studies have shown that localized expression of Dpp in clones of cells can partly rescue a *dpp* mutant phenotype in the wing<sup>10</sup>. Here we investigated the extent to which a constitutively active, ligand-independent form<sup>26</sup> of the Dpp receptor Tkv<sup>27-29</sup> can rescue the *dpp* mutant phenotype when expressed in the endogenous *dpp* domain. The activated receptor is referred to as Tkv\*. Expressing *UAS-tkv\** in a *dpp* mutant background under the control of a *dpp-Gal4* driver significantly restores growth and patterning of the mutant wing (Fig. 5a–d). Note that expression of

FIG. 2 Requirement of *omb* activity for development of a central domain of the wing. **a**, Expression domain of *omb* in the adult wing revealed by expressing *UAS-yellow<sup>+</sup>* under *omb-Gal4* control. Expression of yellow<sup>+</sup> is strong in the centre of the wing and decreases both anteriorly, between veins 1 and 2, and posteriorly, between veins 4 and 5. Arrows indicate the furthest limit of expression. **b**, Expression domain of *omb* in a 30-h pupal wing revealed by expressing *UAS-GFP<sup>+</sup>* under *omb-Gal4* control. Veins 2, 3 and 4 are indicated. **c**, The *omb* phenotype in a female wing of genotype *omb<sup>Ga14</sup>/omb<sup>282</sup>*. The central region of the wing, corresponding to the region of high-level *omb* expression, is deleted. Only females can be trans-heterozygous because *omb* is on the X chromosome. **d**, **e**, Clones of cells homozygous mutant for *omb<sup>282</sup>* in female wings. Mutant cells are marked by the recessive cell-autonomous bristle and trichome marker *forked*. The cell marker cannot be seen at this magnification, so the limits of the clone on the dorsal surface of the wing are indicated by the purple line, and on the ventral surface by the green line. Cells from the line to the near edge of the wing are mutant. **d**, Clone in the anterior compartment. Cells in the distal region of the wing are deleted but, more proximally, mutant cells remain. The interpretation of position with respect to the A/P axis is complicated by wing curvature. For example, spacing between veins 1 and 2 is along the A/P axis in the disc, but appears to be a proximal–distal difference in the adult wing. **e**, Clone in the posterior compartment. Mutant tissue is lost in the distal region of the wing around vein 4, but mutant cells remain between vein 5 and the margin. **f**, Wing from a pharate adult male mutant for *omb<sup>282</sup>*. The wing is not fully inflated because the dead animal was rescued from the pupal case. The central region of the wing is deleted. Similar results on the *omb* mutant and



clonal phenotypes have recently been reported<sup>19</sup>. **METHODS.** *omb<sup>282</sup>* is described<sup>35</sup>. *omb<sup>Ga14</sup>* is a weak mutant allele caused by insertion of a *Gal4* enhancer detector at the *omb* gene. Clones were induced in larvae of genotype *omb<sup>282</sup> forked<sup>369</sup>/M(1) o<sup>89</sup>* by irradiation at  $60 \pm 12$  h with 1,500 Rad of X-ray (115 kV, 5 mA, dose rate  $300 \text{ Rad min}^{-1}$ ). *UAS-GFP* is described<sup>36</sup>. *UAS-yellow<sup>+</sup>* will be described elsewhere (M.C. and G. Morara, manuscript in preparation).

FIG. 3 Different responses of Spalt and *omb* to overexpression of Dpp. **a**, Overlapping domains of Spalt (brown) and *omb-lacZ* (dark blue) expression in a wild-type wing disc; the *omb* domain is broader. **b**, **c**, Overexpression of Dpp in its normal domain causes expansion of the wing along the A/P axis. **b**, Spalt expression domain (brown) is expanded relative to the stripe of *dpp* expression (dark blue), in a disc of genotype *UAS-lacZ/+; dpp-Gal4/UAS-dpp*. **c**, Double labelling shows that the expanded Spalt domain is approximately coextensive with the *omb-lacZ* domain (dark blue) in a disc of genotype *omb-lacZ; dpp-Gal4/UAS-dpp*. This observation has been confirmed by double-label immunofluorescence (data not shown).



**METHODS.** Expression of *omb-lacZ* and *dpp-lacZ* were visualized by X-gal staining followed by antibody staining to visualize Spalt; *dpp-Gal4* has been described<sup>37</sup>.



the *dpp-Gal4* driver depends on *hedgehog* activity rather than *dpp* activity. Therefore the *dpp-Gal4* driver directs expression of the activated receptor in anterior cells adjacent to the compartment boundary in *dpp* mutant discs. Because anterior and posterior compartments do not mix, we anticipated that the activated receptor would direct Spalt and *omb* expression exclusively in anterior cells. Double labelling with antibody to the *engrailed* protein, a marker of posterior cell identity, confirms this prediction (Fig. 5e, f).

The domains of Spalt and *omb* expression produced in this way are not identical; the *omb* domain is significantly broader, filling the rescued anterior wing pouch (Fig. 5e, g), whereas Spalt expression is more restricted to a region near the A/P compartment boundary (arrow, Spalt single-channel label; Fig. 5f). Double labelling for Spalt and *omb* in such wings shows that this results in a nested expression of Spalt (yellow nuclei, arrow; Fig. 5g) within the broader *omb* domain (red), as in the normal wing. These observations indicate that the nested pattern of Spalt and *omb* expression found in wild-type discs cannot depend solely on the local concentration of the secreted Dpp.

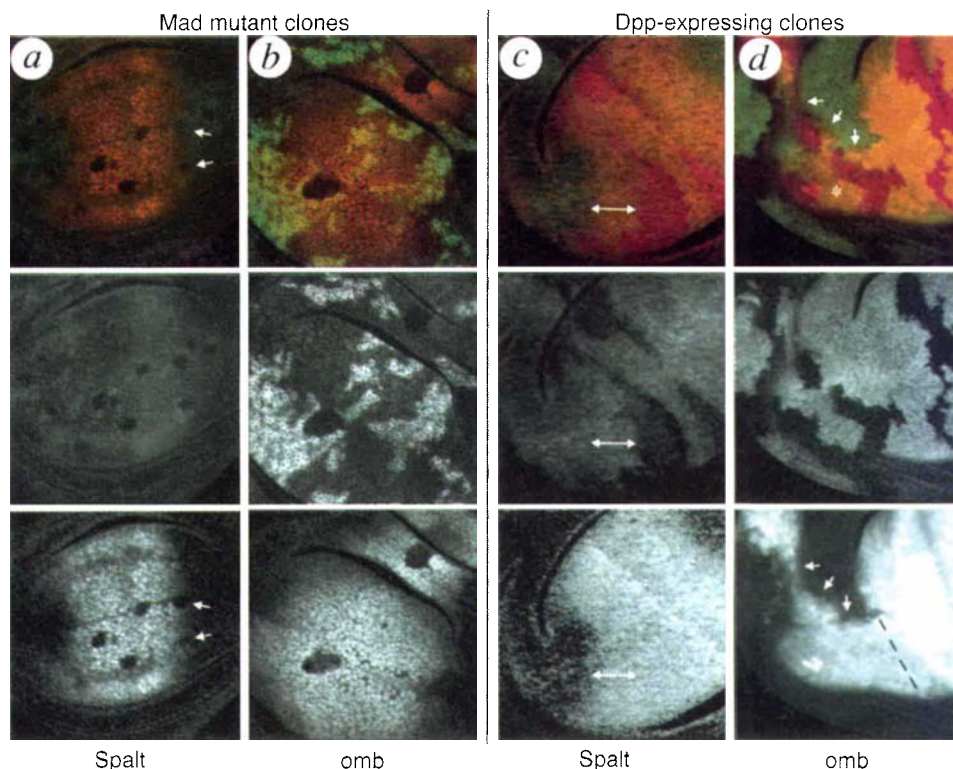
The broader distribution of *omb* with respect to the localized expression of the activated receptor could be explained if cells in which the Dpp pathway was activated relayed a second signal to activate *omb* in nearby cells. To test this possibility, we produced genetically marked clones of cells expressing Tkv\*. Both Spalt and

*omb* are expressed cell autonomously in such clones (Fig. 6a, b), excluding the possibility that the difference between Spalt and *omb* expression (Fig. 5e–g) depends on a downstream relay signal. Clones of Tkv\*-expressing cells can cause overgrowth of wing tissue that consists predominantly of cells in which the activated receptor is expressed (Fig. 6c), again suggesting that these cells do not cause extensive pattern reorganization in the surrounding tissue.

### Relative width of *omb* and Spalt domains

How does the *omb* domain come to be broader than the Spalt domain? Under normal circumstances the cells expressing *dpp* are related by position, not by cell lineage. Their proximity to the A/P boundary brings them in range of the Hedgehog signal, which activates *dpp* expression<sup>12,13</sup>. In the artificial system described here, the activated Dpp receptor is expressed in an equivalent domain under control of the *dpp-Gal4* driver. Double labelling with *UAS-CD2* shows that Spalt expression is restricted to cells expressing the activated receptor (data not shown), whereas the *omb* domain is significantly broader (Fig. 5g). Because we have shown that clones of Dpp-expressing cells activate *omb* over a shorter range than Spalt (Fig. 4), and because there is no signal relay downstream from activation of the Dpp pathway (Fig. 6), we conclude that *omb-lacZ* expression must persist after cells are displaced out of the domain of *dpp-Gal4* expression owing to

FIG. 4 Secreted Dpp directly defines the width of the Spalt domain. a, Clones of cells mutant for a hypomorphic allele of *Mad* do not express Spalt (red). *Mad* mutant cells are marked by the absence of a *lacZ* reporter gene (green). Single-channel images are shown below. Loss of Spalt expression is independent of clone position. Arrows indicate clones near the edge of the Spalt domain. b, *Mad* clones reduce, but do not completely eliminate, *omb-lacZ* expression (red) in the wing pouch. Mutant cells are marked by the loss of the N-myc reporter gene (green); wild-type clones with two copies of N-myc appear bright green. Many wild-type clones are not accompanied by mutant clones owing to the decreased viability of *Mad* mutant cells. Clones mutant for a stronger allele that further reduces *Mad* activity do not survive (not shown). Cell viability is also severely reduced in cells mutant for the Dpp receptor *punt* (not shown). Expression of *omb-lacZ* is reduced in mutant cells, even though the clone is near the centre of the *omb* domain. A comparably sized clone in the wing hinge region lacks *omb* expression, indicating that persistent expression in the clone in the wing pouch is not due to  $\beta$ -Gal stability. Smaller clones induced at a later age show the same effects. It is possible that late removal of Dpp signal cannot lead to complete loss of *omb* expression because cells in the wing pouch continue to express the gene after they have had the signal. Alternatively, the reduction of *Mad* activity by the hypomorphic allele used might be insufficient to support Spalt activation, but is sufficient to support a reduced level of *omb*. This would be consistent with *omb* being sensitive to a lower level of Dpp than is required for Spalt activation; however, it is not compatible with the observation that Spalt is activated farther from Dpp-expressing clones than is *omb*. c, d, Spalt and *omb-Gal4* expression induced by clones of Dpp-expressing cells. Clones of Dpp-expressing cells are marked by the absence of  $\beta$ -GAL (green; see single-channel images below). c, Spalt (red) is induced in Dpp-expressing cells and in cells away from the clones. Arrows denote the range of Spalt activation relative to the clone in all three panels. d, *omb* (red) is induced in Dpp-expressing cells and non-autonomously in nearby cells, but over a shorter range than Spalt. Small arrows indicate regions where *omb* is expressed outside the Dpp-



expressing clones, but only in nearby cells. Asterisk, *omb* expression in cells nested between two clones of Dpp-expressing cells; dashed line, approximate edge of endogenous *omb* domain; magnification of d is slightly greater than c. METHODS. *Mad* clones were made using the hypomorphic allele *Mad*<sup>1,2</sup>. For labelling with anti-Spalt, larvae were of genotype *yHSFLP*; *Mad*<sup>1,2</sup> *FRT40A/arm-lacZ* *FRT40A*. For detection of *omb-lacZ*, larvae were of genotype *yHSFLP/omb-lacZ*; *Mad*<sup>1,2</sup> *FRT40A/N-myc* *FRT40A*. *Mad* clones were induced by heat-shock treatment at 37 °C for 45 min in larvae 72 ± 12 h old. Dpp-expressing clones were produced by FLP-mediated excision of a *> lacZ >* flip-out cassette from a *Ubx > lacZ > dpp* transgene on chromosome 3. Expression was directed using a *Ubx* promoter fragment<sup>38</sup>. For labelling with anti-Spalt, larvae were of genotype *yHSFLP*; *Ubx > lacZ > dpp/+*. For detection of *omb-GAL4*, larvae were of genotype *yHSFLP/y w omb-GAL4*; *UAS-GFP B1/Ubx > lacZ > dpp*.



growth of the disc. Although we have not directly examined the distribution of the *omb* protein in this experiment, two lines of evidence suggest that the reporter gene accurately reflects the expression of the endogenous gene product. First, the spatial domains of the *omb-lacZ* and *Omb* expression are comparable in wild-type discs<sup>19</sup>. Second, we observe rescue of structures from the anterior wing, particularly in the wing margin, that depend on *omb* activity, but not *spalt* activity (Fig. 5d; ref. 20). This rescue cannot be explained if the observed pattern of *omb-lacZ* expression resulted from persistence of  $\beta$ -Gal due to protein stability.

Taken together, our observations suggest that the broad domain of *omb* expression cannot be due to sensitivity to a lower threshold concentration of Dpp for *omb* activation. Rather, they suggest that *omb* is likely to be activated closer to the source of the Dpp signal than is *Spalt*. Given that the domain of *Spalt* is defined by the range of the Dpp signal, the broad domain of *omb* expression could be generated by persistence of *omb* expression in the progeny of cells in which the gene was activated at an earlier time. A demonstration that this is possible comes from the experiment where both genes are initially activated in the same cells by the activated receptor (Fig. 5), but which none the less produces an *omb* domain broader than the *Spalt*

domain (illustrated in Fig. 6). We note that this model does not distinguish between stability of the proteins and maintenance of expression of the transcript. The *omb* transcript is in fact expressed in a broader domain than the *Spalt* transcript, but the same results could be achieved simply by comparing proteins of different stability.

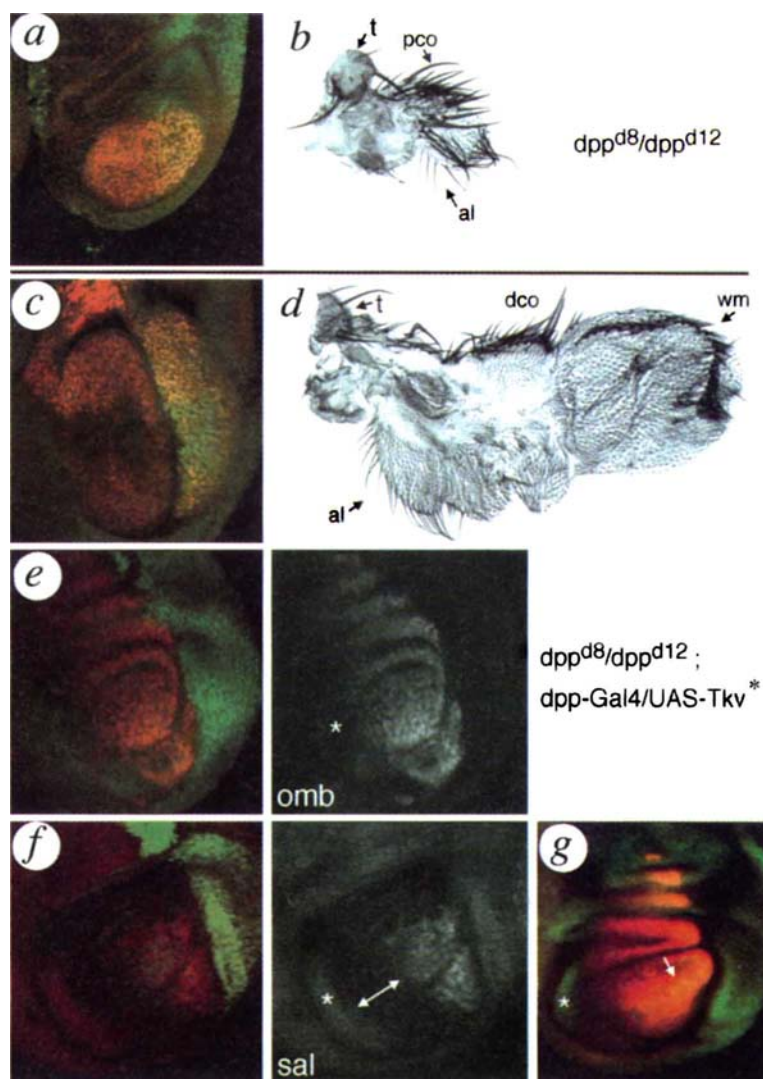
## Two modes of long-range patterning by Dpp

Localized expression of Dpp in cells near the A/P compartment boundary appears to specify cell fates along the A/P axis of the wing by defining nested patterns of gene expression. Here we have considered whether the local concentration of Dpp is sufficient to define the expression domains of two target genes, *spalt* and *omb*. The domain of *Spalt* expression is defined directly by the local concentration of secreted Dpp. However, expression of *omb* appears to be controlled differently. Although these results do not exclude the possibility that Dpp could specify more than one cell fate in a concentration-dependent manner, they clearly indicate that the predictions of a simple morphogen model are not fulfilled for one of the two target genes analysed here.

We have presented evidence for an alternative way of generating long-range pattern, based on the observation that a domain of gene expression (and therefore of fate specification) may be

FIG. 5 Rescue of *dpp* mutant wing by expression of the activated Dpp receptor. **a**, *dpp<sup>88</sup>/dpp<sup>12</sup>* mutant wing disc labelled for Nubbin (red) and Engrailed (green) expression; the wing pouch is small. **b**, Almost no wing tissue remains in *dpp<sup>88</sup>/dpp<sup>12</sup>* flies, except for structures of the proximal hinge region<sup>39</sup>. The proximal costa (pco) remains in the anterior compartment, and a reduced alula (al) in the posterior; t, tegula. **c**, **d**, Partial rescue of the wing by introducing *UAS-tkv<sup>\*</sup>/dpp-Gal4* into the *dpp<sup>88</sup>/dpp<sup>12</sup>* mutant. **c**, Wing disc labelled as in **a**. Both anterior and posterior compartments are larger. The larger posterior compartment corresponds to wing hinge structures (**d**) which express *Spalt* (**f**, **g**). **d**, The wing pouch is significantly restored in *dpp<sup>88</sup>/dpp<sup>12</sup>* flies in which the activated receptor was expressed in cells along the anterior side of the A/P compartment boundary: *UAS-tkv<sup>\*</sup>/dpp-Gal4*. The distal costa (dco) is restored along with a stretch of anterior wing margin (wm). Rescue of the posterior compartment is much more limited. The alula is restored, and other hinge structures are slightly increased. Judging by the asymmetric shape of the wing and the absence of significant posterior margin, the increase in the posterior compartment may be indirectly due to effects on the anterior compartment. In wild-type wings, the activated receptor causes no perturbation of wing morphology in ~80% of flies; ~20% of flies show broadened vein 3 and nicks at the distal tip of the wing, perhaps due to overactivation of the Dpp pathway. **e**, *omb* expression (red) in a *dpp<sup>88</sup>/dpp<sup>12</sup>*; *UAS-tkv<sup>\*</sup>/dpp-Gal4* disc is exclusively anterior, and fills the anterior wing pouch, as in wild-type discs. The asterisk in the single-label channel (right) indicates a lateral region of the wing disc where *omb* is not expressed, corresponding to presumptive wing hinge, as visualized by the lateral domain of *Spalt* expression (asterisk in **g**). The fold that normally separates the lateral domain of *Spalt* expression from the wing pouch does not form in mutant discs (compare **g** with Fig. 1). **f**, *Spalt* expression (red) in a *dpp<sup>88</sup>/dpp<sup>12</sup>*; *UAS-tkv<sup>\*</sup>/dpp-Gal4* disc does not expand to fill the rescued anterior wing pouch. The double-headed arrow in the single-label channel (right) indicates the gap between rescued *Spalt* expression and the lateral (*dpp*-independent) domain of *Spalt* (asterisk; compare with Fig. 1). Comparison with a *UAS-CD2* reporter shows that the *Spalt* domain coincides with cells in which the receptor is activated in this genotype (data not shown). **g**, Double label of *Spalt* (green) and *omb-lacZ* (red) in a *dpp<sup>88</sup>/dpp<sup>12</sup>*; *UAS-tkv<sup>\*</sup>/dpp-Gal4* disc. The overlap of *Spalt* and *omb* expression in cells near the A/P boundary appears yellow. *omb* expression expands to fill the territory between the compartment boundary and the lateral domain of *Spalt* expression (asterisk).

**METHODS.** Constitutively active Dpp receptor, *Tkv<sup>\*</sup>*, was prepared by mutation of Glu 253 in the GS domain to Asp<sup>26</sup>. *tkv<sup>\*</sup>* was cloned into the pUAST vector<sup>40</sup> to prepare *UAS-tkv<sup>\*</sup>*. *tkv<sup>\*</sup>* was expressed under control of the *dpp-Gal4* driver. To generate flies of genotype *dpp<sup>88</sup>/dpp<sup>12</sup>* and *dpp<sup>88</sup>/dpp<sup>12</sup>*; *UAS-tkv<sup>\*</sup>/dpp-Gal4*, combinations of chromosomes 2 and 3



were balanced using T(2,3) Sm6a-Tm6b, Tb. Larvae were selected by the absence of the dominant marker Tb. Antibodies: rat anti-Nubbin<sup>41</sup> mouse 4D9 anti-Engrailed<sup>42</sup>; rabbit anti- $\beta$ -gal was used to visualize *omb-lacZ* in **e**, and mouse anti- $\beta$ -gal was used with rabbit anti-*Spalt* in **g**; mouse anti-CD2 (Serotec). *UAS-CD2* (ref. 43).

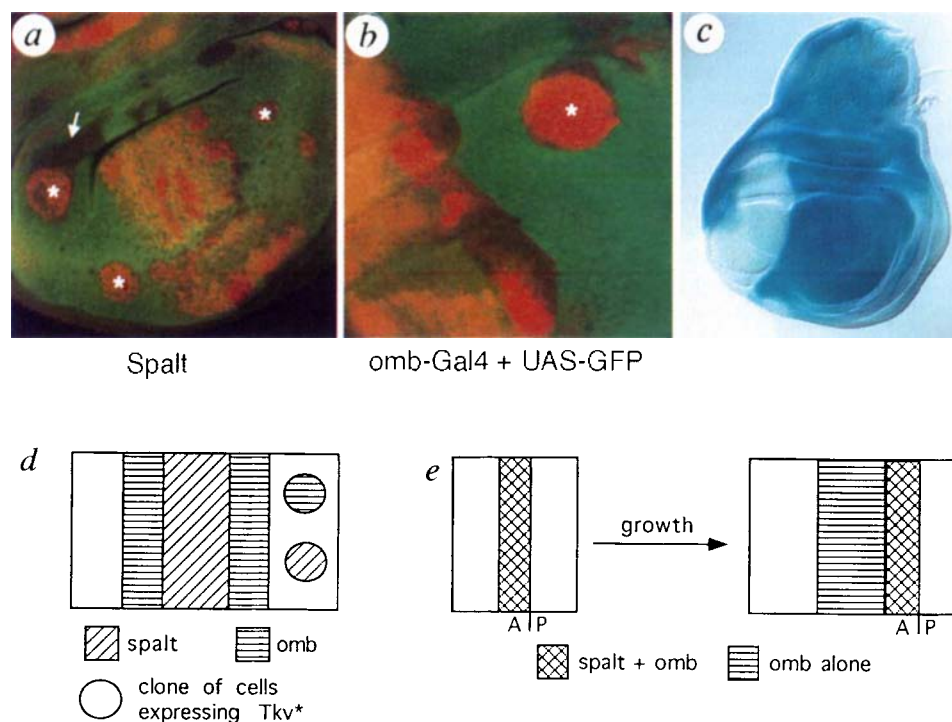


FIG. 6 Cell-autonomous activation of Spalt and *omb* in clones of cells expressing the activated Dpp receptor. *a, b*, Clones are marked by the loss of  $\beta$ -Gal (green). *a*, Spalt (red) is turned on in clones of cells expressing *tkv\**, but not in surrounding wild-type cells. There is no overlap in the red and green labels at the edges of the clone. Overlap of the two labels in the endogenous Spalt domain appears yellow–orange. Some clones outside the endogenous Spalt domain are indicated (asterisks); one large clone extends out of the wing pouch (arrow). Spalt is expressed autonomously in the portion of the clone that lies in the wing pouch, but not in cells of the wing hinge. This mirrors the restriction of the endogenous Spalt domain to the wing pouch, and suggests that a transcription factor on which the Dpp pathway acts to activate Spalt may be restricted to the wing pouch. Clones were visualized using rabbit anti-Spalt and mouse anti- $\beta$ -Gal in discs of genotype *y HSF1p; Ubx > lacZ > tkv\**. *b*, Cell-autonomous expression of *omb-Gal4* (red) in clones of cells expressing *tkv\**. There is no overlap in expression of *omb-Gal4* in wild-type cells surrounding the clone (asterisk). Overlap of the two labels in the endogenous *omb* domain appears yellow–orange. A second large clone lies at the edge of the endogenous domain. The edge of the clone defines the boundary of the expression domain. *omb* expression was visualized by UAS–GFP expression under control of *omb-GAL4* (red channel) in disc of genotype *yw omb-GAL4/y HSF1p; Ubx > lacZ > tkv\*/UAS-GFP B1*. *c*, Overgrowth in wing caused by a clone of *Ubx > tkv\** cells that is visualized by absence of  $\beta$ -Gal expression (unlabelled cells in a blue background); the overgrowth consists almost entirely of cells lacking the  $\beta$ -Gal reporter and expressing *tkv\**. Presence of the clone induces limited compensatory overgrowth in the surrounding wild-

type tissue. *d*, Schematic representation of cell-autonomous activation of Spalt and *omb* in clones of cells expressing the activated receptor *Tkv\**. Clones are indicated by circles outside the endogenous expression domain. All cells in a clone are progeny of a single cell in which the *lacZ* flip-out cassette was excised, allowing *Tkv\** expression. The absence of reporter-gene expression in wild-type cells surrounding the clone shows that the domains of expression are not defined by relay of a second signal, induced in cells where the Dpp pathway is activated. Activation of Dpp by the Hedgehog pathway, as in clones of *pka* mutant cells<sup>44</sup> is an example of signal relay. *e*, Schematic representation of the rescue of anterior wing fates by localized activation of *Tkv\** using the GAL4 system (Fig. 5c–g). Although *omb* and Spalt can only be activated in cells expressing *Tkv\** (*a, b*), the domain of *omb* expression becomes broader than that of Spalt. Unlike the experiment in *a, b*, cells expressing *Tkv\** under control of *dpp-GAL4* are not a clonal population. As the disc grows, progeny of the cells move out of range of the Hedgehog signal and lose expression of the *dpp-GAL4* driver and hence of *Tkv\**. Spalt expression is restricted to cells expressing *Tkv\** in this system (data not shown). Comparison with Spalt indicates that *omb* expression must persist in cells that have moved out of range of the Hedgehog signal, and so have lost expression of *Tkv\**. This mechanism is sufficient to generate the nested pattern of Spalt and *omb* expression in the absence of secreted Dpp.

**METHODS.** Expression of *Tkv\** in clones was done under control of a *Ubx* promoter fragment, followed FLP-mediated excision of a *> lacZ >* flip-out cassette from a *Ubx > lacZ > tkv\** construct.

defined by the history of a cell at least as much as by its direct responsiveness to secreted signals. Furthermore, we have shown that the organization of pattern within the wing disc can be reproduced to a significant extent through local activation of the Dpp signalling pathway using a ligand-independent activated Dpp receptor in a *dpp* mutant background; this result cannot be explained by the morphogen model.

A third way in which long-range pattern can be generated involves local induction followed by signal relay (for example, induction of Dpp expression by Hedgehog at the compartment boundary). Although we have shown that this is not the case for regulation of Spalt and *omb* by Dpp, there is evidence for signal relay in D/V axis formation in the *Drosophila* leg. Spatially localized expression of secreted Wingless can cause axis duplication in the fly leg, which has led to the suggestion that Wingless may function as a morphogen<sup>30</sup>. However, subsequent studies

showed that cell-autonomous activation of the Wingless signal-transduction pathway in clones of cells faithfully mimics the non-autonomous patterning effects attributed to Wingless in the D/V axis, casting doubt on the role of Wingless as a morphogen in the leg<sup>25</sup>.

In conclusion, we suggest that the elaboration of pattern in multicellular fields may depend on different processes, only some of which are based on information conferred by the local concentration of secreted signalling molecules. □

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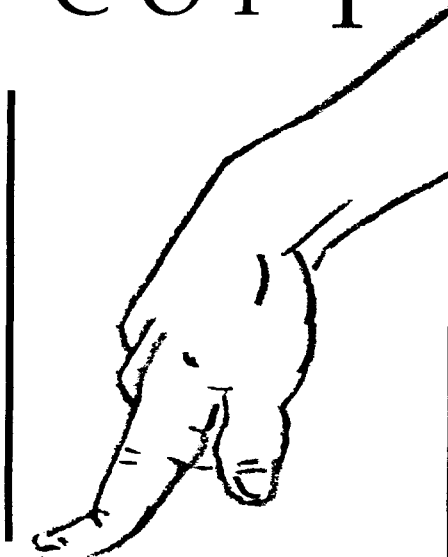
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# Constraints from Galileo observations on the origin of jovian dust streams

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THE *Ulysses* spacecraft detected streams of sub-micrometre-sized dust particles as it approached Jupiter in 1992<sup>1,2</sup>. Although interplanetary space was known to contain dust, the presence of discrete streams was completely unexpected. The directions from which the dust grains struck the spacecraft strongly suggested that the source lay somewhere within the Jupiter system. Three origins were proposed, the comet Shoemaker–Levy 9 (ref. 3), Jupiter's gossamer ring<sup>4</sup>, and the volcanoes on Io<sup>5</sup>, but there was no definitive evidence for or against any of the options. Here we report the detection by the *Galileo* spacecraft of even more intense dust streams—including three intense dust storms of month-long duration, with impact rates up to 10 times higher than those observed by *Ulysses*. Our analysis of the data confirms that the dust streams originate near Jupiter; we are able to rule out a cometary origin, but cannot yet determine conclusively whether the dust comes from Io or the ring.

*Galileo* was launched in 1989, and followed a looping trajectory through the Solar System that brought it to Jupiter on 7 December 1995. The *Galileo* dust detector<sup>6</sup>, like its twin aboard *Ulysses*<sup>7</sup>, is an impact ionization detector which detects the plasma cloud released when a dust grain strikes its sensor. The impact direction (rotation angle) is determined by the spin position of the spacecraft at the time of impact, and both the impact speed and the mass of the dust grain can be derived from characteristics of the recorded charge pulses<sup>8</sup>. Normally these data are all transmitted to ground, but because *Galileo* relies on its low-gain antenna for communications and data transfer (its high-gain antenna failed to deploy), it cannot always transmit data in real time and must store it on-board. Because of limited memory aboard the spacecraft, some dust data is overwritten before it can be transmitted. Impacts are recorded in counters, but the complete information (from which the mass, velocity and direction are derived) is often lost. Thus for *Galileo*, we have two types of data: (1) impact rates and (2) information on individual dust impacts (impact charge, particle mass, relative velocity, impact time and impact direction).

In July 1994, the *Galileo* instrument was re-programmed to

improve its noise suppression capability; this increased its mass sensitivity by a factor of eight which is critical for studies of dust streams as these particles are very small. Figure 1a shows the rate of small impacts observed during the two years before *Galileo*'s arrival at Jupiter. We call the three largest peaks dust 'storms' and the smaller peaks dust 'streams' and list relevant parameters in Table 1. The *Galileo* dust streams are similar in duration and intensity to the streams observed by *Ulysses*, although they do not display the one-month periodicity that the *Ulysses* streams did. Dust storms are an order of magnitude longer and more intense than dust streams. During the third and most intense dust storm, special campaigns were run by the *Galileo* operations team at the Jet Propulsion Laboratory that provided daily data transmissions and, in a special case (5 October 1995), hourly transmissions for 10 successive hours. These data showed that the impact rate fluctuated by up to a factor 100 over a day. No significant fluctuations have been found in the hourly sequence primarily because, at that time, the impact rate was low and statistical variations prevailed. No major changes were noted in the dust impact rate when *Galileo* crossed the bow shock in front of the jovian magnetosphere at the end of November.

Directional information on impacts is displayed in Fig. 1b. Impacts are separated in two ranges of impact charge. Small impacts are concentrated both in time and in direction (towards Jupiter). The threshold that effectively separates Jupiter dust-stream impacts from most of the rest of the impacts was apparent in the *Ulysses* data<sup>2</sup>. Impact charges of dust-stream particles are most frequently very small, just above the detection threshold, suggesting even more numerous impacts with smaller amplitudes. Impact speeds range from 10 to 40 km s<sup>-1</sup> and masses range from 10<sup>-16</sup> to 10<sup>-13</sup> g. The true mass and speed values of the impacts may deviate significantly from those derived because the impact signals are both close to the threshold and at the edge of the calibrated range.

Zook *et al.*<sup>9</sup> analysed *Ulysses* dust-stream measurements by taking into account both actual solar-wind plasma and magnetic-field data. They calculated dust trajectories between Jupiter and

## BOX 1 Potential sources of jovian dust streams

### (1) Comet Shoemaker–Levy 9<sup>3</sup> (SL9)

**Characteristics.** Short-term dust emission during the June 1992 breakup of SL9 near Jupiter and the June 1994 impact of SL9 into the atmosphere of Jupiter. At an emission speed of 100 km s<sup>-1</sup> dust should have reached *Galileo* within 10 days of the impact.

**Related observations.** No coincidence between dust streams and main SL9 events: some streams occurred before and others long after these events.

### (2) Jupiter's gossamer ring<sup>4</sup>

**Characteristics.** Continuous generation of ejecta from mutual collisions of ring particles, and from impacts of grains in the jovian system and interplanetary meteoroids. No fast source modulations. Modulations of the emitted dust intensity should occur only during the transit of the jovian magnetosphere and interplanetary space.

**Related observations.** Dust-stream activity is highly time variable, whereas *Galileo* measurements of the interplanetary magnetic field show little difference between the periods when a stream occurs and when no stream occurs.

### (3) Volcanoes on Jupiter's satellite Io<sup>5</sup>

**Characteristics.** Time-variable dust emission correlated with volcanic activity. From *Voyager* observations of volcanic plumes a particle size range 0.001–0.01 µm was derived<sup>13</sup>. A dust production rate of 10<sup>5</sup> to 10<sup>8</sup> kg s<sup>-1</sup> is estimated.

**Related observations.** Dust-stream activity is highly time variable. Periodic stream activity was observed by *Ulysses*, but is not obvious in *Galileo* data. Because of lack of frequent Io observations, no correlation with volcanic activity has been identified.

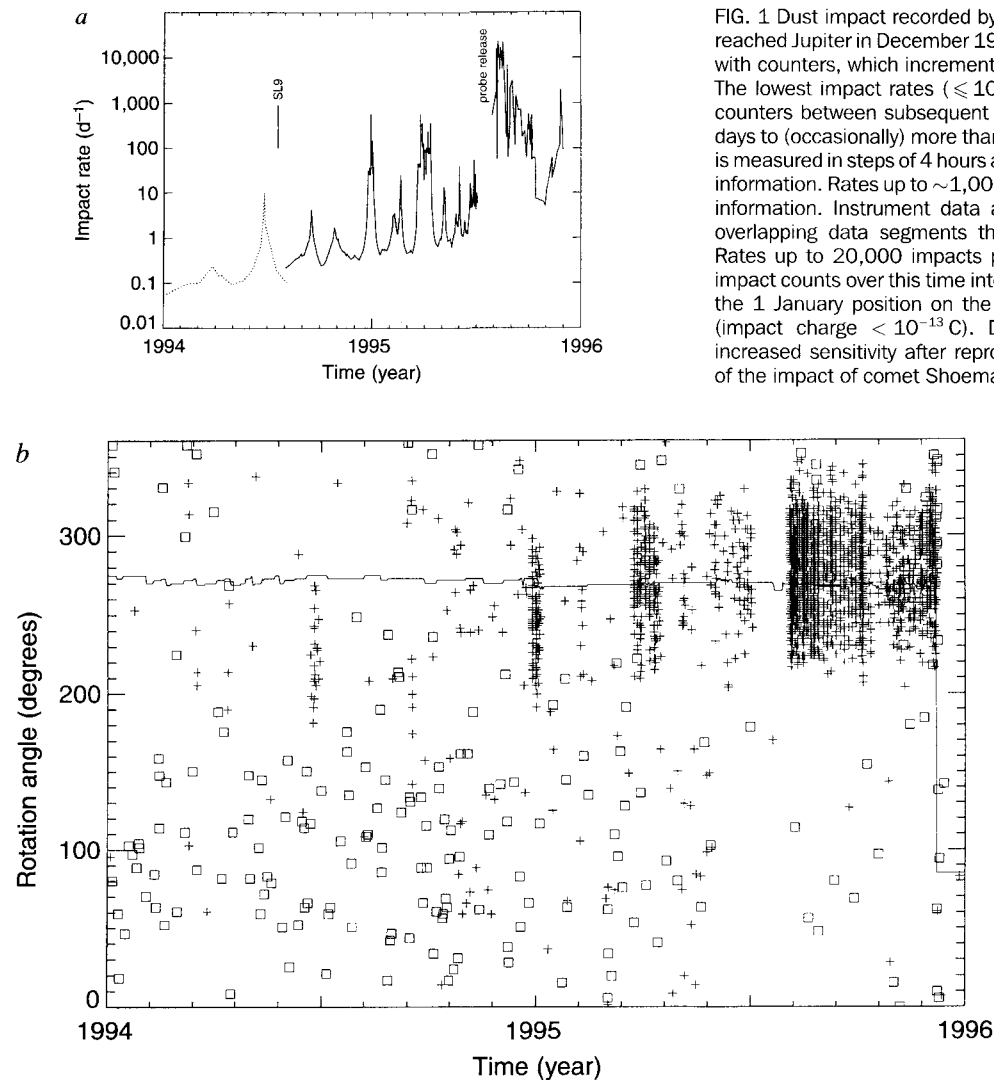


FIG. 1 Dust impact recorded by Galileo during the last two years before it reached Jupiter in December 1995. The instrument measures impact rates with counters, which increment from 0 to 255 over varying time intervals. The lowest impact rates ( $\leq 100 \text{ d}^{-1}$ ) are determined from differences in counters between subsequent data transmissions, extending from a few days to (occasionally) more than a month. The time of an individual impact is measured in steps of 4 hours and is transmitted with the complete impact information. Rates up to  $\sim 1,000$  impacts per day can be resolved from this information. Instrument data are transmitted to ground in two sets of overlapping data segments that are separated by about 20 minutes. Rates up to 20,000 impacts per day can be determined by comparing impact counts over this time interval. Note that year numbers are shown at the 1 January position on the horizontal axis. *a*, Rate of small impacts (impact charge  $< 10^{-13} \text{ C}$ ). Dotted line, initial sensitivity; solid line, increased sensitivity after reprogramming the dust instrument. The time of the impact of comet Shoemaker-Levy 9 (SL9) onto Jupiter is indicated.

During July 1995, no data were received near the time of release of the atmospheric probe. *b*, Spacecraft rotation angles. Rotation angle is taken to be  $0^\circ$  when the dust-sensor axis points closest to the north ecliptic pole. At rotation angle  $90^\circ$ , the detector-axis points parallel to the ecliptic plane roughly in the direction of planetary motion. Impacts are marked according to their impact charges; crosses,  $< 10^{-13} \text{ C}$ ; squares,  $\geq 10^{-13} \text{ C}$ . The rate of big impacts is about  $0.4 \text{ d}^{-1}$  and is only slowly varying<sup>14</sup>. The solid line indicates the direction to Jupiter. Data that include impact directions were obtained for only a small fraction of the impacts that occurred during dust storms.

TABLE 1 Parameters of the Jupiter dust streams observed by Galileo

| Stream number | Centre of stream (date) | Distance to Jupiter ( $R_J$ ) | Duration (d) | No. of counted impacts | No. of impacts with complete information |
|---------------|-------------------------|-------------------------------|--------------|------------------------|------------------------------------------|
| G1            | 25 June 94              | 3,500                         | 8            | 22*                    | 21                                       |
| G2            | 16 Sept. 94             | 3,000                         | 0.2          | 7                      | 6                                        |
| G3            | 1 Nov. 94               | 2,770                         | 10           | 16                     | 16                                       |
| G4†           | 31 Dec. 94              | 2,390                         | 10           | $\sim 600$             | 148                                      |
| G5            | 7 Feb. 95               | 2,160                         | 5            | 18                     | 13                                       |
| G6†           | 3 April 95              | 1,800                         | 21           | $\sim 1,000$           | 169                                      |
| G7            | 5 May 95                | 1,600                         | 12           | 26                     | 21                                       |
| G8            | 13 June 95              | 1,340                         | 40           | 145                    | 90                                       |
| G9†           | 7 Sept. 95              | 755                           | 80           | $\sim 400,000$         | 1,131                                    |
| G10           | 16 Nov. 95              | 220                           | 20           | $\sim 5,000$           | 51                                       |

All impacts were counted, but the complete information was received on the ground for only a small number of impacts (see text). Jupiter radius  $R_J = 71.4 \times 10^3 \text{ km}$ .

\* Reduced sensitivity.

† Dust storm.

Ulysses that matched the observed impact directions, and argued that only very fast ( $> 200 \text{ km s}^{-1}$ ) particles with a charge-to-mass ratio  $\sim 1,000 \text{ C kg}^{-1}$  fit the Ulysses observations. Such a charge-to-mass ratio can only be reached by dust particles as small as  $0.01 \mu\text{m}$  in radius. This size is about a factor of 10 below the size which we derived from the measured impact parameters and it implies that

Jupiter dust-stream particles are outside the calibrated mass and impact speed ranges.

Figure 2 top panel shows the mean spacecraft rotation angle of the instrument during the time of the dust storms. The mean rotation angle of the various impacts measures the out-of-ecliptic component of the dust velocity, the component that is most



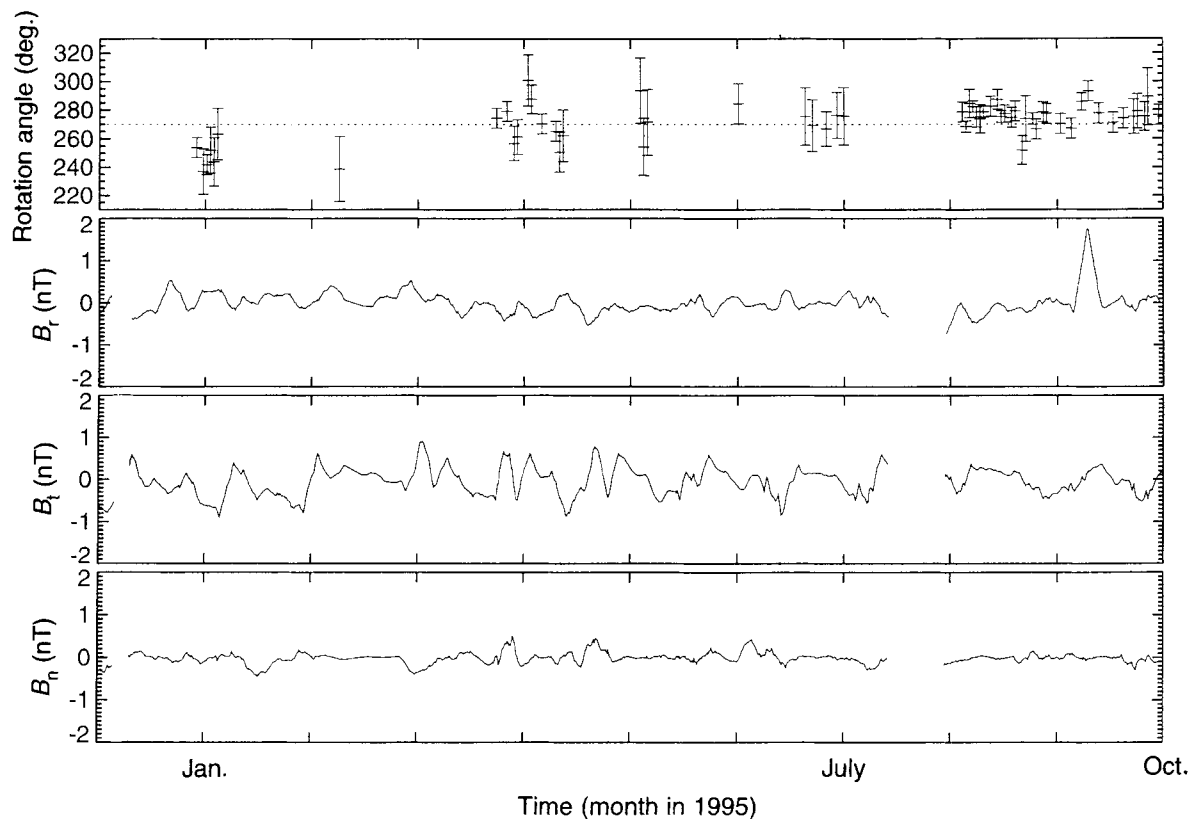


FIG. 2 Comparison of dust impact directions with magnetic field components over the period of the dust storms. Upper panel, mean spacecraft rotation angle of small dust impacts (impact charge  $< 10^{-13}$  C) recorded during the time of the dust storms. The mean value is given whenever at least 4 impacts per day occurred. The dotted line indicates the direction to Jupiter. Lower panels, interplanetary magnetic field averaged over 4 days before the time given. The radial (with respect to the Sun),  $B_r$ , tangential,  $B_t$ , and normal (with respect to the solar equator),  $B_n$ , components are given. The data gap in July 1995 occurred when the atmospheric probe was released. Submicrometre-sized charged dust particles interact

with the interplanetary magnetic field via the Lorentz force which is perpendicular to both the velocity and the magnetic field vector. The velocity with which a dust particle travels relative to the magnetic field is roughly opposite to the solar-wind motion which carries this field away from the Sun. The dominant component of the interplanetary magnetic field in the outer Solar System is the tangential component,  $B_t$ , and, therefore, the Lorentz force acts predominantly away from or towards the solar equator (which is  $7.3^\circ$  off the ecliptic plane), that is, this force is directed approximately up- or downwards relative to the ecliptic plane (at rotation angles  $90^\circ$  and  $270^\circ$ ).

affected by the tangential component of the interplanetary magnetic field. We show the average components of the magnetic field<sup>10</sup> on the same timescale for comparison (Fig. 2 lower panels). The deviation of the rotation angle from the Jupiter direction resembles the fluctuations of the tangential magnetic field,  $B_t$ , around the zero value especially during the April storm. The magnetic field did not vary much during the time of the September storm, nor did the mean rotation angle of the dust particles. An accurate calculation of the interplanetary trajectories for the Galileo dust streams is underway.

Jupiter dust-stream activity seems to be highly time variable. The intensity varied by at least a factor of 1,000 between the weakest and the strongest streams observed by Galileo and Ulysses. Whereas streams recorded by Ulysses showed a periodicity of about 28 days, we do not find any correlation with this period in the Galileo data. Although the magnetic field was especially low during the last Galileo storm, the magnitude and variation was comparable to the magnetic field during the Ulysses post-flyby streams at other periods, for example, during the April storm.

Three potential dust sources have been discussed in the literature: the breakup and impact of comet Shoemaker–Levy 9<sup>3</sup>, Jupiter's gossamer ring<sup>4</sup>, and the volcanoes on Jupiter's satellite Io<sup>5</sup>. In Box 1 we show the characteristics of the three potential sources and discuss the relevant observations. Because of the missing coincidence of major stream activity with Shoemaker–

Levy 9 events, we rule out with high probability this comet as the cause for the dust streams. Despite the spectacular display of the comet impact itself, the production of dust capable of leaving the Jupiter system is most probably too low<sup>3</sup> to make a significant contribution to dust-stream activity.

Observations of Jupiter's gossamer ring show that it consists mainly of micrometre-sized particles located in a disk between 1.8 and 3.0 jovian radii. Collisions among ring particles are common, and the submicrometre-sized products of these collisions are whisked away from Jupiter by powerful electromagnetic forces<sup>4</sup>. As this source produces a nearly steady flux of escaping particles, the periodicity in the Ulysses streams and the modulation of the Galileo streams would need to be effected solely by electromagnetic forces in the jovian magnetosphere and solar wind.

Sub-micrometre-sized dust produced during volcanic activity on Io<sup>5</sup> can reach the jovian magnetosphere<sup>11</sup> and is subsequently ejected into interplanetary space by electromagnetic forces<sup>5</sup>. The dust production rate from Io's volcanic activity is assumed to be highly variable, and hence dust streams need not be modulated so strongly by electromagnetic forces. Unfortunately, observations of Io are still too sparse to establish or rule out a direct correlation between volcanic eruptions and dust-stream activity.

We conclude that the jovian ring and volcanoes on Io remain viable sources, but rule out dust from Shoemaker–Levy 9. More information on the temporal variation of dust streams and their source will be gained starting in July 1996, when we resume

receiving data from Galileo. In 2002, when the Cassini spacecraft flies by Jupiter *en route* to Saturn, we will get the first compositional measurements of the dust grains, which will provide more clues to their origin<sup>12</sup>. □

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## Direct observation of a surface charge density wave

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A CHARGE density wave (CDW) is a periodic symmetry-lowering redistribution of charge within a material, accompanied by a rearrangement of electronic bands (such that the total electronic energy is decreased) and usually a small periodic lattice distortion<sup>1,2</sup>. This phenomenon is most commonly observed in crystals of reduced symmetry, such as quasi-two-dimensional<sup>3</sup> or quasi-one-dimensional<sup>4</sup> materials. In principle, the reduction of symmetry associated with surfaces and interfaces might also facilitate the formation of CDWs; although there is some indirect evidence for surface charge density waves<sup>5–12,14</sup>, none has been observed directly. Here we report the observation and characterization of a reversible, temperature-induced CDW localized at the lead-coated (111) surface of a germanium crystal. The formation of this new phase is accompanied by significant periodic valence-charge redistribution, a pronounced lattice distortion and a metal–nonmetal transition. Theoretical calculations confirm that electron–phonon coupling drives the transition to the CDW, but it appears that some other factor—probably electron–electron correlations—is responsible for the ground-state stability of this phase.

Pb/Ge(111)- $\alpha$  consists of 1/3 monolayer of equivalent lead adatoms spaced  $\sim 7\text{Å}$  apart in a hexagonal array of  $T_4$  sites atop the bulk-truncated germanium lattice, forming the  $(\sqrt{3} \times \sqrt{3})R30^\circ$  arrangement shown in Fig. 1. Clean, well ordered germanium (111) surfaces were first prepared in ultrahigh vacuum, followed by a room-temperature lead dose and subsequent anneal (at  $\sim 250^\circ\text{C}$ ) to produce  $\alpha$ -phase<sup>15–19</sup>. Coverage was monitored with both low-energy electron diffraction (LEED) and Auger electron spectroscopy. In practice, some Ge adatom defects were always present in the overlayer.

We employed three experimental surface analysis techniques to characterize this thin film. LEED was used to determine the symmetry of the surface atomic lattice. Inelastic electron scattering (electron energy-loss spectroscopy (EELS)) was used to probe the excitation spectra of our interface. Scanning tunnelling microscopy (STM) was used to image the spatial variations in the surface charge density with atomic resolution<sup>20</sup>.

We present first our results acquired at room temperature, where all previous investigations were performed<sup>15–19,21</sup>. LEED data (Fig. 2a) include several sharp diffraction spots, characteristic of the  $(\sqrt{3} \times \sqrt{3})R30^\circ$  surface symmetry displayed in Fig. 1. EELS data (Fig. 2a) indicate that the room-temperature  $\alpha$ -phase

is metallic. This result is consistent with simple electron counting, where (based on the Pauli principle) two electrons from each unit cell sequentially populate the electronic bands (starting from low energy). In any geometry with an odd number of electrons per unit cell, the uppermost occupied band will be only partially filled, resulting in a metallic structure. The  $(\sqrt{3} \times \sqrt{3})R30^\circ$  surface unit cell contains one lead atom (with a valency of four) and three outer layer germanium atoms (each with one unpaired electron), totalling seven valence electrons in all, and is therefore expected to be metallic. Figure 3a presents room-temperature STM data acquired in the same scan but at different bias voltages. In both the filled state and empty state, a hexagonal array of identical protrusions can be seen. The results of previous experiments<sup>18</sup> and computer simulations<sup>19</sup> indicate that each protrusion imaged is a lead adatom.

As the sample temperature is lowered, properties of the  $\alpha$ -phase exhibit a dramatic change. For surfaces with a low enough defect density, the low-temperature LEED pattern now shows the presence of extra spots (Fig. 2b), characteristic of a new  $(3 \times 3)$  symmetry. This transition in structure is gradual and reversible, with an onset near  $-20^\circ\text{C}$ . EELS data of the low temperature  $\alpha$ -phase are likewise very different from their room-temperature counterparts. There is no continuum of low energy losses; instead, a discrete loss onset can be seen, indicating a semiconducting interface. The inset of Fig. 2b shows similar data for a non-zero parallel momentum transfer (angle of reflection  $\neq$  angle of incidence) indicating more clearly the onset value. Whereas the room temperature  $\alpha$ -phase is metallic, our observations indicate that a small band gap ( $E_g \leq 0.065\text{ V}$  at 100 K) evolves as tempera-

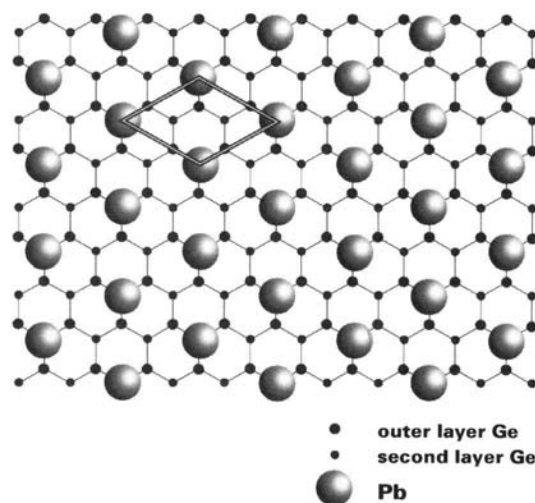


FIG. 1 Ball model of the  $\alpha$ -phase of Pb/Ge(111); coverage is 1/3 monolayer. Lead adatoms are  $7.0\text{Å}$  apart on  $T_4$  sites atop the bulk truncated germanium lattice. The indicated unit cell shows the interface's  $(\sqrt{3} \times \sqrt{3})R30^\circ$  symmetry (relative to the bulk truncated  $(1 \times 1)$  symmetry).

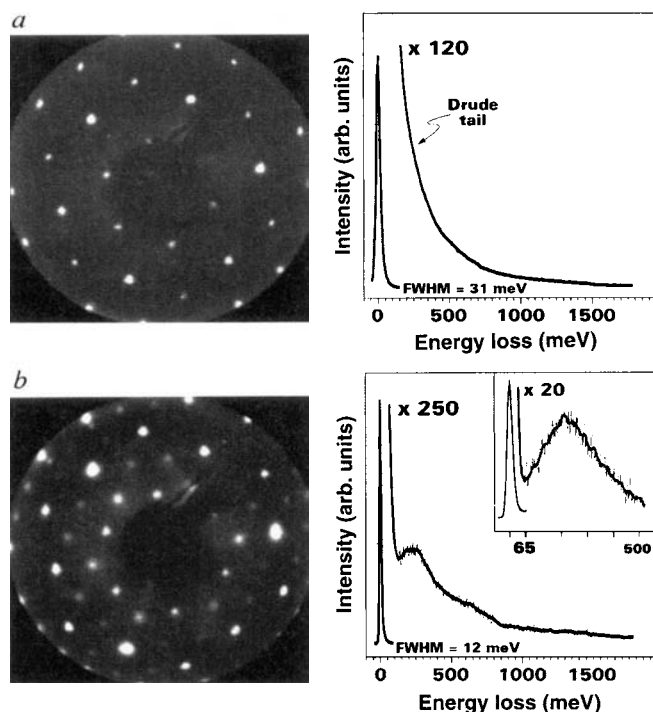


FIG. 2 Room-temperature (a) and low-temperature (b;  $T \approx 100$  K) LEED (left) and EELS (right) data from the  $\alpha$ -phase; beam energy for LEED was 48.6 eV. In LEED, the change in symmetry from the room-temperature ( $\sqrt{3} \times \sqrt{3}$ )R30° structure to the low-temperature ( $3 \times 3$ ) structure is highlighted by the emergence of many new diffraction maxima. At room temperature, EELS indicates that  $\alpha$ -phase is metallic by (1) the presence of a broad, slowly decreasing Drude tail, produced when electrons at the Fermi level are excited into the adjacent continuum of empty states, and (2) the absence of a discrete energy loss onset. The discrete onset in the low-temperature spectrum, created when electrons are excited with minimum excitation energy into the unoccupied bands, indicates a surface band gap. The energy onset of such a loss spectrum corresponds to the gap magnitude  $E_g$ . Inset, off-specular data (non-zero parallel momentum transfer) indicating that the ground state band gap is no more than 65 meV.

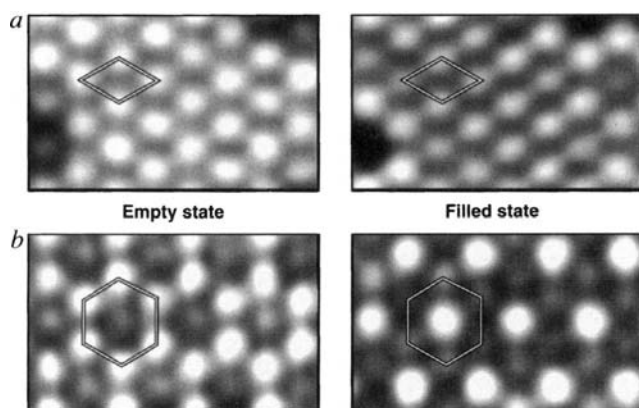


FIG. 3 Room-temperature (a) and low-temperature (b;  $T \approx 60$  K) STM data from the  $\alpha$ -phase. Empty- and filled-state images were acquired with sample bias  $V_s = +1$  V (electrons tunnelling out from the sample) and  $-1$  V (electrons tunnelling into the sample) respectively. Their registry is assured, that is, each point in the empty-state image is produced from the same real space coordinate as the corresponding point in the filled-state image. At room-temperature, each lead adatom appears identical, whereas at low temperature, a honeycomb-pattern charge rearrangement is evident. Repeat units are identified in each image.

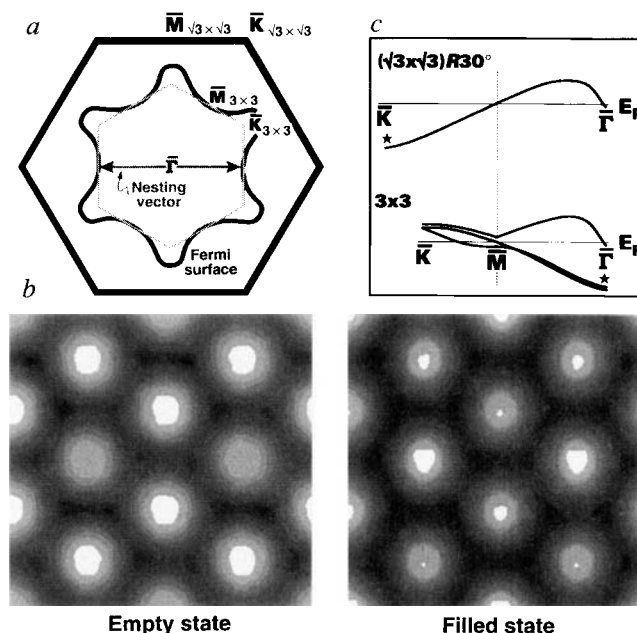


FIG. 4 Calculations of electronic structure were performed for several supercells with various  $k$ -point samplings. During the calculations of the low-temperature phase, the outermost germanium layers and the lead layer were allowed to relax. a, The wavy black line inside the outer hexagon (first Brillouin zone) is a curve drawn through calculated Fermi surface points for the ( $\sqrt{3} \times \sqrt{3}$ )R30° structure. The illustrated nesting vector is  $1/\sqrt{3}$  the length of a reciprocal lattice vector, matching the new ground-state symmetry of ( $3 \times 3$ ); the new Brillouin zone is indicated in grey. b, Calculated contours of constant integrated density of states (between  $E_F$  and  $\pm 1$  eV) for the fully relaxed structure, showing the observed ( $3 \times 3$ ) symmetry and honeycomb-pattern charge rearrangement. c, Calculated band structure along high-symmetry directions for both the ( $\sqrt{3} \times \sqrt{3}$ )R30° and ( $3 \times 3$ ) structures. Although the former structure is metallic with a single band crossing midway between the zone centre ( $\Gamma$ ) and the zone boundary ( $K$ ), a small gap opens between two bands at this location (the new M point) in the SCDW phase, while a third crosses  $E_F$ . This gap is localized in a small area of the surface Brillouin zone and, therefore, the calculated outer layer density of states near  $E_F$  shows practically no change between the room-temperature and low-temperature structures. Note that the two filled bands from  $\Gamma$  to M in the ( $3 \times 3$ ) structure are roughly those expected from folding back the ( $\sqrt{3} \times \sqrt{3}$ )R30° band approaching  $K$  (stars denote equivalent  $k$ -space locations). For both structures, valence bands disperse about 0.6 eV.

ture is lowered. Our EELS study suggests that this electronic transition also occurs gradually and reversibly, but at a slightly lower onset temperature than the structural transition.

It is the change in STM imaging at low temperatures, however, that elucidates the nature of the observed transition. Low-temperature filled- and empty-state images of  $\alpha$ -phase are shown in Fig. 3b. Unlike the room-temperature results, Pb adatoms no longer appear equivalent, and the same ( $3 \times 3$ ) symmetry found in low-temperature LEED data can be seen. This is not the result of a rippled topography. Rather, it reflects the sensitivity of the STM technique to the spatial profile of occupied (filled-state image) and unoccupied (empty-state image) electronic states. One out of three Pb adatoms now possess an enhanced relative concentration of filled states (and appears brighter in the filled-state image). This same atom also possesses a diminished relative concentration of empty states (and appears darker in the empty-state image). The result is a honeycomb-patterned charge superstructure commensurate with the underlying lattice. As the sample temperature increases, this new charge superstructure gradually subsides and the sample returns to the equivalent adatom configuration of room temperature.

What we observe experimentally is now clear. On decreasing temperature, the Pb/Ge(111)- $\alpha$  system forms a commensurate



surface charge density wave (SCDW) independent of any bulk phenomena. This transition occurs gradually and reversibly with an onset just below room temperature. As with many examples of bulk CDWs, this surface rearrangement of valence charge incorporates a structural distortion. Comparison of our LEED data to that of other systems suggests the lead atom plane is significantly rumpled at low temperature, probably of the order of  $\sim 0.2$  Å. The measured value of the SCDW band gap,  $\sim 0.065$  V, corresponds well with the observed critical temperature,  $\sim -20^\circ\text{C}$ , via the mean-field CDW relationship,  $E_g = 3.53 K_B T_c$  (ref. 22). The value of the tiny SCDW band gap measured by EELS could not be reliably verified using STM (as is generally possible<sup>23</sup>) in our case owing to signal-to-noise problems at the Fermi energy ( $E_F$ ).

The only major inconsistency with the simple SCDW picture results from electron counting. The new low-temperature ( $3 \times 3$ ) unit cell is three times as large as the room-temperature ( $\sqrt{3} \times \sqrt{3}$ )  $R30^\circ$  cell, containing three lead and nine germanium atoms and, therefore, 21 valence electrons. This is again a sufficient condition for metallicity, in stark contradiction to the semiconducting ground state inferred from the EELS data.

In an attempt to identify the driving force for stabilization of a SCDW, we performed first-principle density functional calculations<sup>24</sup> for this system using the local density approximation. The calculations confirm that the room-temperature phase should be metallic with a half-filled band of surface states positioned in the gap region of the germanium substrate. In Fig. 4a, the wavy black line inside the ( $\sqrt{3} \times \sqrt{3}$ )  $R30^\circ$  surface Brillouin zone (the outer hexagon) depicts the shape and location of the calculated two-dimensional Fermi surface. A vector of constant momentum transfer connecting parallel segments of Fermi surface, called a nesting vector, is identified. The electronic response function of a nested Fermi surface is strongly peaked at twice the Fermi wavevector ( $2k_F$ ), which facilitates enhanced electronic screening of phonons (quantized lattice vibrations) with momentum  $2k_F$ . This screening results in a soft phonon at  $2k_F$ , called a Kohn anomaly. If the nesting (and hence the screening) is strong enough, a static lattice distortion can occur, incorporating the symmetry of the nesting vector. As seen in Fig. 4a, the predicted nesting vectors match the symmetry of the observed ( $3 \times 3$ ) ground state (inner hexagon, Fig. 4a). Therefore, theory predicts that Fermi-surface nesting, or more generally electron-phonon coupling, drives this transition.

When not constrained in our calculation, lead and surface germanium atoms rearrange themselves spontaneously in a ( $3 \times 3$ ) structure, reducing the total energy by about 0.020 eV per ( $\sqrt{3} \times \sqrt{3}$ )  $R30^\circ$  unit cell. The corrugation of the lead adlayer is  $\sim 0.05$  Å. Two simulated STM images for this structure (Fig. 4b) show remarkable qualitative agreement with the experimental data. Figure 4c shows the calculated band structure near the Fermi energy for both the ( $\sqrt{3} \times \sqrt{3}$ )  $R30^\circ$  and ( $3 \times 3$ ) structures. At room-temperature, a single half-filled band (with strong lead  $p_z$  character) crosses the Fermi energy. Owing to the reduced symmetry, three bands exist in this region at low temperature:

one crosses the Fermi energy (metallic) as before, but the other two show the effect of the new SCDW periodicity, opening up a small gap at the new ( $3 \times 3$ ) zone boundary (M). However, problems still exist. First, the calculated distortion of the lead atoms is not large enough to account for the intensity of the fractional-order LEED spots. Second, and most importantly, the theoretical ( $3 \times 3$ ) surface is metallic, contrary to our EELS data. But the apparent shortcomings of theory might in fact indicate the true nature of the ground state. We infer that electron-electron interactions (not properly accounted for in the local density approximation) result in larger-than-predicted lattice displacements, as well as the formation of a surface band gap (a Mott insulator) which further stabilizes the low-temperature phase. This is a reasonable inference, as calculated charge density plots for Pb/Ge(111) indicate the states involved with Fermi surface nesting are localized, a necessary precursor to the onset of significant correlation effects. The role of electron correlation in CDW formation and stability was first discussed by Overhauser<sup>25</sup>.

Although most of the bulk quasi-two-dimensional CDWs are driven purely by a bond reorganization, our SCDW is reminiscent of that for the layered compound 1T-TaS<sub>2</sub> (refs 26–28). In this system, lowering sample temperature initiates a reversible transition in the CDW phase from an incommensurate structure to a commensurate one, accompanied by the sharp evolution of a pseudo-gap (again contrary to electron counting). But in contrast to Pb/Ge(111)- $\alpha$ , TaS<sub>2</sub> Mott localization has been considered only an accidental consequence of the CDW distortion and was not considered responsible for its stability.

Although other proposed examples of CDWs at surfaces exist, Pb/Ge(111) is thus far unique. For example, surfaces of the layered transition metal dichalcogenides<sup>28</sup> (including TaS<sub>2</sub>) possess a charge superstructure, but only as the termination of bulk CDWs. The reconstruction of semiconductor surfaces on cleaving in vacuum is sometimes referred to as a CDW<sup>1,2</sup>, but these transitions are irreversible in the experimentally accessible temperature range, involve large structural distortions, and can be accounted for by covalent bond rearrangement. On other surfaces including W(100) and Mo(100) (refs 5–11), and alkali-metal covered Cu(111) (ref. 12), CDWs have been speculated to account for certain experimental data, but have not been directly observed. A Peierls distortion<sup>13</sup>, the one-dimensional analogue of a CDW, has likewise been proposed to explain anomalous observations of Ti/Cu(110) (ref. 14).

It is likely that other unmistakable SCDWs will be found within the wealth of known metal-semiconductor interfaces. For example, aluminum, indium, gallium, lead and tin all produce T<sub>1</sub> adatom  $\alpha$ -type structures on Si(111) at room temperature. A determination of which systems undergo the SCDW transformation will lend further insight into the physical processes responsible for this phenomenon. It might even be possible to 'engineer' the Fermi surface by co-deposition of different adatom species, producing a range of both commensurate and incommensurate low-temperature structures. □

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# Synthesis and structure of a layered titanosilicate catalyst with five-coordinate titanium

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TITANIUM occurs widely in the Earth's crust, traces of it being present in most rocks, soils and clays. It is estimated that titanosilicate minerals alone number more than 100; but, remarkably, in only one of these does the Ti(IV) ion take up five-fold coordination. This is in fresnoite<sup>1</sup> ( $\text{Ba}_2\text{TiSi}_2\text{O}_8$ ), which contains square-pyramidal  $\text{TiO}_5$  polyhedra. In the course of a programme<sup>2–11</sup> to produce new microporous and mesoporous solid catalysts, we have discovered an unusual non-centrosymmetric, tetragonal layered solid ( $\text{Na}_4\text{Ti}_2\text{Si}_8\text{O}_{22} \cdot 4\text{H}_2\text{O}$ ), designated JDF-L1, which promises to have interesting applications in materials chemistry. This material contains five-coordinate Ti(IV) ions in the form of  $\text{TiO}_5$  square pyramids in which each of the vertices of the base is linked to  $\text{SiO}_4$  tetrahedra [ $\text{TiO} \cdot \text{O}_4(\text{SiO}_3)_4$ ] to form continuous sheets. The structure was solved by applying *ab initio* methods to data obtained by X-ray absorption spectroscopy and powder X-ray diffraction. The interlamellar  $\text{Na}^+$  ions of JDF-L1 are replaceable by protonated amines, and after treatment with a mixture of dilute acid and hydrogen peroxide the parent solid selectively oxidizes phenol to quinone. These results indicate that the material should have useful catalytic, intercalation and ion-exchange properties analogous to those of aluminosilicate clays.

Open-structure (microporous as well as mesoporous) crystalline silicas, in which some of the Si(IV) ions have been isomorphously replaced by Ti(IV) ions, are often very good catalysts and are of prime significance in the petrochemical industry. Among the most widely known is the so-called TS-1 titanosilicate introduced by the Enichem Co.<sup>12</sup> for the catalytic conversion of phenol to hydroquinone and the epoxidation of styrene. Titanium-substitution in the framework of Zeolite- $\beta$ <sup>13</sup>, as well as in many other microporous<sup>12,14</sup> and mesoporous silicas<sup>11,15–17</sup>, results in good catalytic activity and selectivity in several other important reactions as has recently been discussed by Corma<sup>13</sup> and others<sup>16</sup>. Whereas Ti(IV) is present in these solids (when dry) in tetrahedral coordination, there are other titanium-containing microporous materials in which the Ti(IV) ions are in six coordination<sup>18–21</sup>.

To produce new micro- or mesoporous siliceous solids, an organic template is used which, during the course of hydrothermal

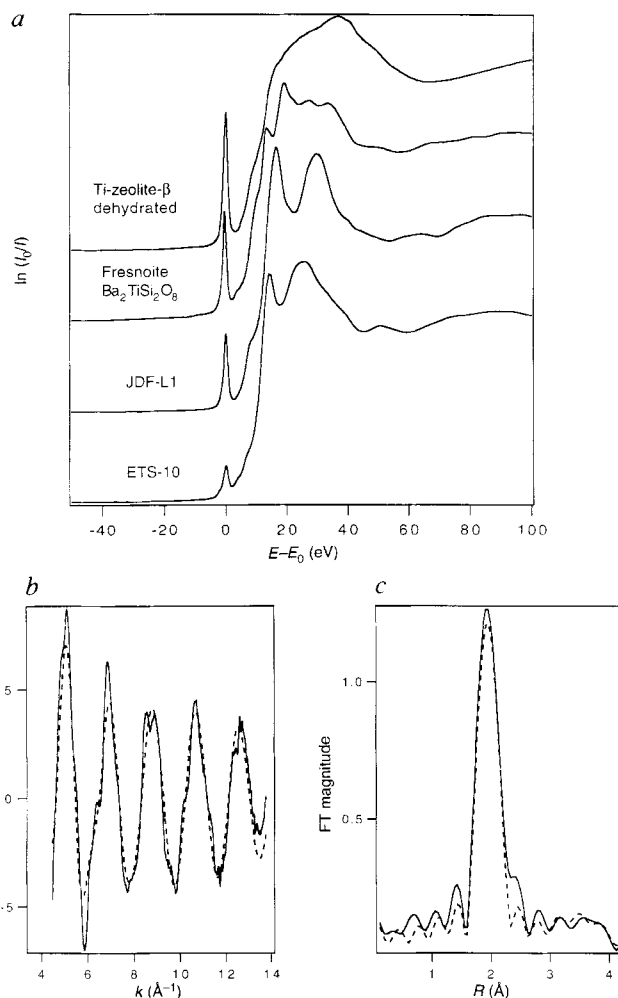


FIG. 1 *a* Ti K-edge X-ray absorption near-edge spectrum of the as-prepared JDF-L1 along with the corresponding spectra of the mineral fresnoite (see text) and relevant Ti-containing zeolitic materials. *b*, The extended X-ray absorption fine structure (EXAFS) of the Ti-K-edge, and *c*, the corresponding Fourier transform of the as-prepared JDF-L1. The data were recorded at station 7.1 of the Synchrotron Radiation Source, Daresbury Laboratory; operating energy was 2 GeV and a typical current of 150 mA, using transmission mode and a Si(111) double crystal monochromator; ion chambers were employed for measuring incident and transmitted beam intensities.

synthesis, steers the process of crystallization—in ways that are still unclear—so as to generate open structures. The organic template is accommodated within the cavities and channels of this open structure, and is subsequently removed from these locations by calcination. Although routines are now so well worked out that one may be confident of repeating the synthesis

TABLE 1 Structural parameters of JDF-L1

| Atom | Site symmetry |     | x         | y         | z         | $B_{\text{iso}} (\text{\AA}^2)$ | Occupancy |
|------|---------------|-----|-----------|-----------|-----------|---------------------------------|-----------|
| Ti   | 2c            | 4.. | 0.5       | 0         | 0.7778(1) | 0.66(3)                         | 2         |
| Si   | 8g            | 1   | 0.2405(2) | 0.6808(2) | 0.6445(1) | 0.60(2)                         | 8         |
| O(1) | 8g            | 1   | 0.2514(4) | 0.4655(4) | 0.6740(2) | 0.89(6)                         | 8         |
| O(2) | 8g            | 1   | 0.3808(4) | 0.7714(4) | 0.7384(3) | 1.23(6)                         | 8         |
| O(3) | 2c            | 4.. | 0.5       | 0         | 0.9373(5) | 0.8(1)                          | 2         |
| O(4) | 4f            | ..2 | 0.2166(4) | 0.2166(4) | 0.5       | 2.2(1)                          | 4         |
| Na   | 4d            | 2.. | 0.5       | 0.5       | 0.8329(2) | 1.82(6)                         | 4         |
| Ow   | 4e            | ..2 | 0.3495(4) | 0.3495(4) | 0         | 1.6(1)                          | 4         |

These parameters were derived from refinement of the model for JDF-L1 using powder X-ray diffraction data (collected with a capillary sample); space group  $\text{P4}_212$ . Estimated standard deviations of refined parameters are in parentheses.  $B_{\text{iso}}$ , isotropic temperature factor.  $R_1 = 7.3\%$ ;  $R_{\text{wp}} = 16.9\%$ ;  $R_{\text{exp}} = 15.6\%$ .

of known, open structures and of designing new ones, occasionally, layered solids, rather than three dimensionally extended open structures, are produced from the templated, precursor gel<sup>2-8,22,23</sup>. Such is the case in the work we report here.

JDF-L1, (Jilin–Davy–Faraday, layered solid no. 1)<sup>9</sup>, of nominal composition  $\text{Na}_4\text{Ti}_2\text{Si}_8\text{O}_{22}\cdot 4\text{H}_2\text{O}$ , was synthesized using tetrabutyl orthotitanate (TBOT, 98%), fumed silica (99%), sodium hydroxide, aqueous hydrogen peroxide (30%), aqueous ammonia (25%) and tetrabutylammonium bromide (TBABr), gels of the following molar compositions being prepared for the synthesis:  $x\text{TiO}_2$ :  $y\text{Na}_2\text{O}$ :  $0.12\text{TBABr}$ :  $2.5\text{H}_2\text{O}_2$ :  $36\text{H}_2\text{O}$ :  $8.5\text{NH}_3$ , where  $x = 0.1\text{--}0.5$  and  $y = 0.3\text{--}0.6$ . Typically, 1.75 g of TBOT was mixed with 12 ml of ammonia, and to the mixture were successively added 6 ml  $\text{H}_2\text{O}_2$  and 0.8 g NaOH. After stirring, a clear yellow solution was formed and to this 0.8 g TBABr and 1.2 g fumed silica were added. The final reaction mixture, with a molar composition of  $\text{SiO}_2$ :  $0.25\text{TiO}_2$ :  $0.48\text{Na}_2\text{O}$ :  $0.12\text{TBABr}$ :  $2.5\text{H}_2\text{O}_2$ :  $36\text{H}_2\text{O}$ :  $8.5\text{NH}_3$  was stirred for about 2–3 hours, followed by heating in a PTFE-lined stainless-steel autoclave at 453 K under autogenous pressure for about 10 days. The crystalline product was filtered, washed with distilled water and dried at ambient temperature, the final product being an off-white microcrystalline powder. Besides TBABr other amines (among them triethylamine, tributylamine, isopropylamine and dimethylamine) could be used for the synthesis of JDF-L1.

The Ti K-edge X-ray absorption spectrum of JDF-L1 (Fig. 1a) reveals the absence of the customary sixfold coordination which is a feature of the vast majority of titaniferous minerals<sup>24</sup>. However, the average Ti–O distance obtained from the analysis of the extended X-ray absorption fine structure (EXAFS) data is much larger than one would have expected from four-coordinated Ti(IV) (ref. 10) and is similar to the Ti–O distance found in the mineral fersnoite<sup>1</sup>. Detailed analysis (Fig. 1b and c) of the first coordination sphere clearly revealed the presence of square-pyramidal coordination around Ti(IV), with the apical oxygen having a short Ti–O distance in line with the pre-edge intensity. The short Ti–O distance is symptomatic of a double bond and the excess framework charge is compensated by two monovalent ions. The chemical analysis indeed reveal Na/Ti ratio of close to 2.

The initial high-resolution X-ray powder diffractogram (not shown), recorded with a flat-plate  $\theta/2\theta$  geometry using synchrotron radiation ( $\lambda = 1.59939(1)\text{\AA}$ ) is distinctly different from that of any known titanosilicate—microporous or otherwise. The 20 reflections used by the indexing program TREOR<sup>25</sup> suggested a tetragonal unit cell. The occurrence of systematic absences led to the choice of two suitable space groups of highest symmetry,  $P4_212$  (no. 90) and  $P4_2m$  (no. 113), yielding the unit-cell dimensions  $a = b = 7.3673(1)\text{\AA}$ ,  $c = 10.6998(1)\text{\AA}$ , unit cell volume  $580.754(7)\text{\AA}^3$  and  $Z = 1$ . After a pattern decomposition was calculated using the program MPROFIL<sup>26</sup> (a Le Bail<sup>27</sup> decomposition program that refines a profile without a structural model), the extracted structure factor amplitudes were put into the direct methods program SHELXS86<sup>28</sup>. This yielded a suitable starting model for Rietveld<sup>29</sup> profile analysis from which it emerged that JDF-L1 was a layered structure. We therefore collected data in capillary mode ( $\lambda = 2.0000(1)\text{\AA}$ ), see Fig. 2, to minimize the effects of preferred orientation. Silicon, titanium and sodium ratios, obtained from the chemical analysis, were used for the refinement of the structure which proceeded smoothly in the space group  $P4_212$  yielding the structure shown in Fig. 3. The kinship of the titanosilicate frameworks of fersnoite<sup>1</sup> and JDF-L1, each with their five-coordinated Ti(IV) ions in the silicate sheets, is highlighted in Fig. 4, where, for clarity, interlamellar cations (and water in the case of JDF-L1) have been omitted. Not only is there good correspondence between the Ti–O distances obtained from two distinct structure-determining approaches—spectroscopy and diffraction—but we also note that the most appropriate way to describe the immediate environment of the Ti(IV) ions is, as in the case of fersnoite,  $\text{TiO}\cdot\text{O}_4$ . In effect, JDF-L1 has double sheets, whereas fersnoite is a single-sheet titanosilicate. The five-

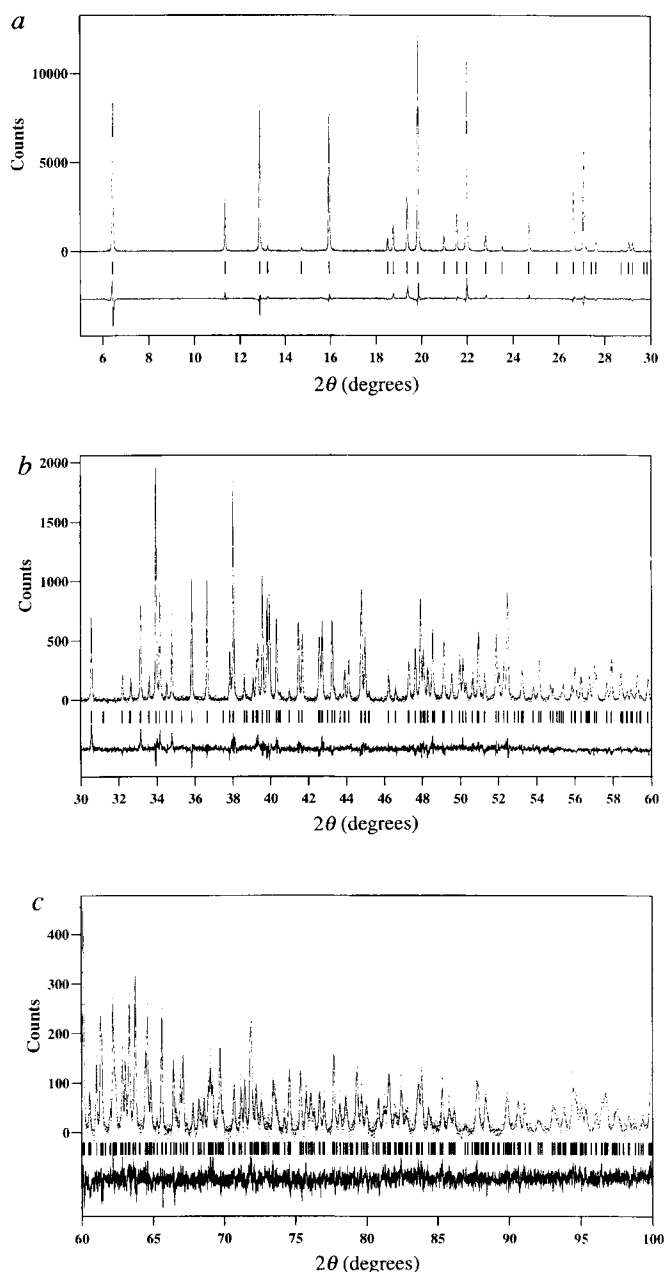


FIG. 2 The synchrotron observed (points) and calculated (line) profile, Bragg X-ray powder reflections (ticks) and difference profile (bottom trace) for JDF-L1, at room temperature, measured at  $1.20000(1)\text{\AA}$ . a, Low  $2\theta$ ; b, intermediate  $2\theta$ ; c, high  $2\theta$ . The flat-plate-derived diffractogram showed significant preferred orientation of the 001 reflections, which could be eliminated when the diffractometric data were collected (at the Daresbury Synchrotron Radiation Source, Station 2.3) using the capillary method (0.5-mm quartz capillary). The model converged giving  $R_1 = 7.3\%$ ,  $R_{wp} = 16.9\%$ ,  $R_{exp} = 15.6\%$ . Good agreement was found between bond distances obtained from X-ray diffraction studies and from Ti K-edge EXAFS analysis (given in parentheses) as follows, resulting in: one Ti–O of  $1.707(5)\text{\AA}$  ( $1.70\text{\AA}$ ); four Ti–O of  $1.946(3)\text{\AA}$  ( $1.96\text{\AA}$ ); four Ti–Si of  $3.350(1)\text{\AA}$  ( $3.37\text{\AA}$ ), and four Ti–Na of  $3.7305(4)\text{\AA}$  ( $3.68\text{\AA}$ ).

membered condensed rings (consisting of four  $\text{SiO}_4$  units and one  $\text{TiO}_5$ ) are comparable in aperture size to those of the five-membered condensed rings (five  $\text{SiO}_4$  units) that occur in ZSM-5, ZSM-11, ZSM-23<sup>30</sup> and a variety of other pentasil<sup>31</sup>. (The apertures are just about large enough to permit diffusion, along the planes, of small molecules such as  $\text{H}_2$ ).



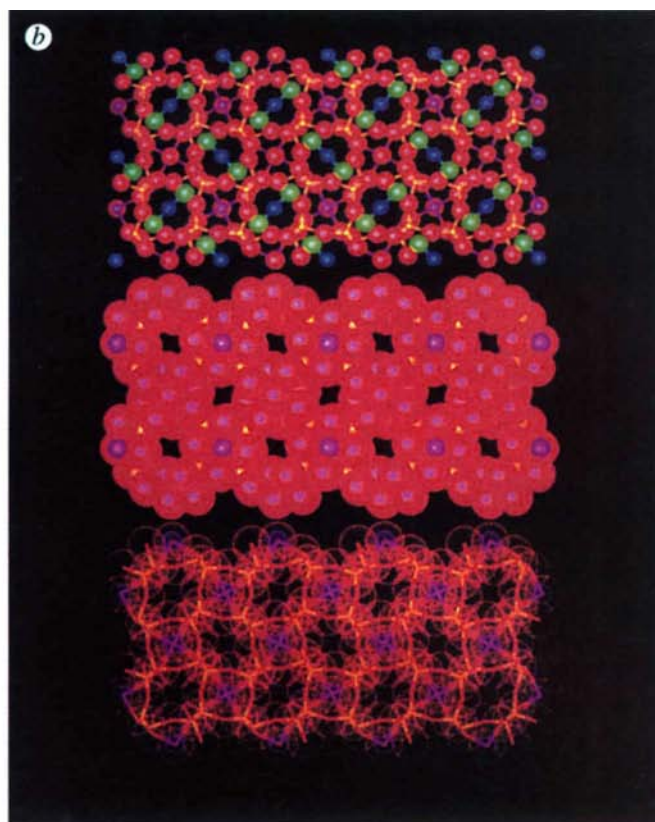


FIG. 3 *a*, Perspective view along the [100] or [010] directions, revealing the layered structure of JDF-L1 (Si, yellow; Ti, purple; framework O, red; Na, blue; water O, green). The layers of this structure run vertically in the Figure. *b*, View perpendicular to the layers, along the [001] direction. The

framework shows an aperture that occupies the central two-fold axis, the Na<sup>+</sup> ions being associated with this aperture. Top, complete structure, ball and stick model. Middle, space-filling model of framework only. Bottom, stick model of framework but with atomic surfaces superimposed.

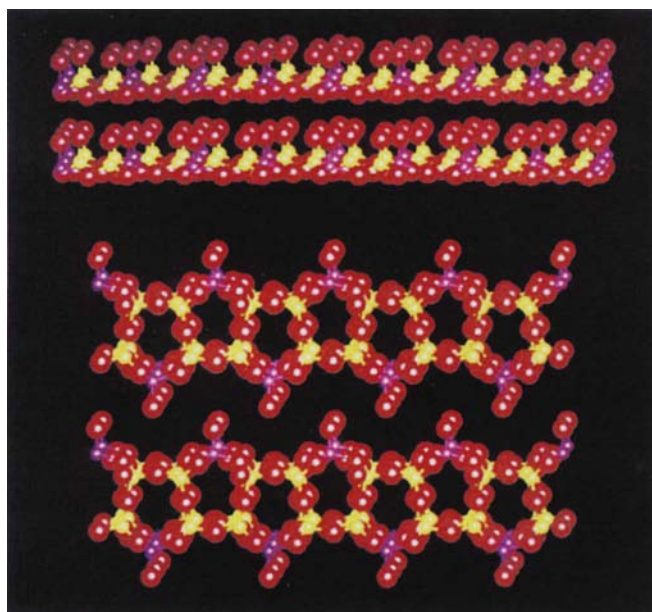


FIG. 4 Comparison of the titanosilicate layers for JDF-L1 (bottom) and fresnoite (top). The exchangeable cations have been omitted for clarity.

Each titanosilicate layer in JDF-L1 is composed of small cage-type units linking together to form a layer comprising two orthogonal channel systems. The five-coordinate titanium pyramidal site points alternately into the flanking layers of interlamellar water and cations. The cages are composed of eight Si and two Ti site, the Ti site being five-coordinated, with four long Ti–O bonds of 1.945(8) Å and one short double bond of 1.63(2) Å. There are only Si–O–Si and Ti–O–Si linkages; there are no Ti–O–Ti connections. Viewed perpendicular to the layers, along the [001], direction the framework shows an aperture (Fig. 3*b*): they occupy the central two-fold axis, the Na<sup>+</sup> ions being associated with this aperture. The Na ions occupy a site on a two-fold axis at 4(1/2, 1/2, *z*) and are six-coordinate, with four oxygen framework linkages (two O1 and two O2) and two water molecules.

Apart from its uniqueness, the structure of JDF-L1 implies, and experiment confirms, that the interlamellar alkali metal ions are exchangeable. Like the kandite and smectite clays, typified by kaolin and montmorillonite, alkylamines in their protonated state may replace the Na<sup>+</sup> ions thereby causing an expansion of the interplanar spacing (from 10.70 to 29.70 Å in the case of nonylamine). The intercalation capacity is found to be four molecules of nonylamine per formula unit of JDF-L1. JDF-L1 in its as-prepared state—unlike several framework-substituted Ti-bearing or Ti-grafted microporous and mesoporous (MCM-41) solids—is inactive in the epoxidation of cyclohexene. But, after treatment with a mixture of dilute HCl and H<sub>2</sub>O<sub>2</sub> (0.6 mmol of HCl and 2 ml of H<sub>2</sub>O<sub>2</sub> (30%) with 0.2 g of JDF-L1) before the addition of a reaction mixture consisting of H<sub>2</sub>O<sub>2</sub>, acetone and phenol (30 mmol H<sub>2</sub>O<sub>2</sub>, 10 ml acetone, 10 mmol phenol), it converts phenol to quinone with 100% selectivity. The conversion is 20% complete after 2 h at 60 °C, whereas as-prepared JDF-L1 and

powdered TiO<sub>2</sub> in the form of anatase are totally inactive. The catalytic properties of JDF-L1 based materials are now under investigation. We note that JDF-L1, because of its non-centrosymmetric tetragonal structure, may have potential as a pyroelectric material and, either in its natural (hydrated) or intercalated state, as a precursor of polar glass ceramic<sup>32</sup>. □

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## Photochemical release of biologically available nitrogen from aquatic dissolved organic matter

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**DISSOLVED organic material in marine and freshwater ecosystems constitutes one of the Earth's largest actively cycled reservoirs for organic matter<sup>1</sup>. The bacterially mediated turnover of chemically identifiable, low-molecular-mass components of this pool has been studied in detail for nearly three decades, but these compounds constitute less than 20% of the total reservoir<sup>2</sup>. In contrast, little is known about the fate of the larger, biologically more refractory molecules—including humic substances—which make up the bulk of dissolved organic matter. Here we report results from bacterial bioassays and photochemical studies indicating that exposure to sunlight causes dissolved organic matter to release nitrogen-rich compounds that are biologically available, thus enhancing the bacterial degradation of humic substances. We demonstrate that ammonium is among**

**the nitrogenous compounds released and is produced most efficiently by ultraviolet wavelengths. Photochemical release of ammonium from dissolved organic matter has important implications for nitrogen availability in many aquatic ecosystems, including nitrogen-limited high-latitude environments and coastal oceans, where inputs of terrestrial humic substances are high.**

Aquatic humic substances are organic acids (of molecular masses between 500 and 10,000), operationally defined on the basis of their retention on hydrophobic resins and further categorized as humic acids or fulvic acids based on their solubility at low pH<sup>2</sup>. Humic substances make up the largest single class of dissolved organic matter (DOM), accounting for 30 to 60% of the DOM in most natural waters<sup>2</sup>. Although abundant, they are considered to be the component of DOM from which all easily available energy has already been extracted, and therefore have received little attention from a microbiological perspective. The accessibility of carbon in humic substances to bacteria may, however, be considerably greater than previously assumed<sup>3,4</sup>. Exposure to sunlight enhances the breakdown of humic carbon to lower-molecular-mass compounds, some of which are assimilated rapidly by natural bacteria<sup>5–10</sup>.

Despite recent advances in our understanding of the biogeochemistry of humic carbon, the fate of the nitrogen bound in aquatic humic substances and its susceptibility to photochemical degradation have yet to be addressed. Humic substances have been considered as unlikely sources of nitrogen for microbial food webs because the biological availability of humic-bound nitrogen is low for both marine and freshwater environments<sup>11,12</sup>. Furthermore, C:N ratios of humic substances are high (averaging 50:1; ref. 2), making it unlikely that they can supply nitrogen to bacterial degraders in the required proportions to carbon for growth, and less likely that bacteria can regenerate ammonium from humic substances for use by autotrophs<sup>13</sup>. This current view of the ecological and biogeochemical roles of humic substances would need to be modified, however, should some significant fraction of humic-bound nitrogen prove to be readily assimilable by planktonic microorganisms.

In a study designed to examine the effects of solar radiation on the biological availability of humic substances, the fulvic acid fraction of DOM isolated by hydrophobic resin<sup>14</sup> from a boreal pond was reconstituted in deionized water at its original concentration (891 µM C) and exposed in quartz flasks to natural sunlight

for four hours. Twelve bioassay treatments were established by additions of inorganic nutrients (N only, P only, or N plus P) and/or a labile carbon source (glucose) to irradiated or non-irradiated fulvic acid solutions. The growth of natural bacterioplankton was followed over the next 92 h as an assay for biological availability. Bacterial growth was low in all fulvic acid solutions without added inorganic P (Fig. 1a, c, e). In the presence of sufficient P, however, growth was significantly enhanced in treatments exposed to sunlight and in those with labile carbon additions (Fig. 1b, d, f). The striking lack of an inorganic nitrogen effect on biological availability suggests that the fulvic acids themselves provided sufficient

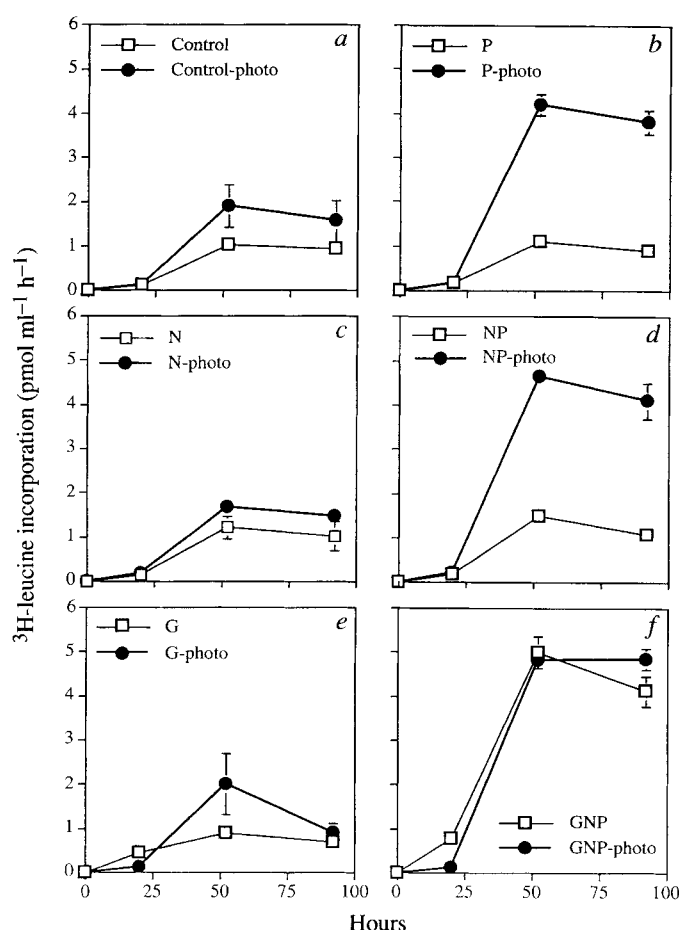


FIG. 1 Bacterial bioassay to determine biological availability of boreal pond fulvic acids. A fulvic acid concentrate was isolated by XAD-8 resin without cation exchange<sup>14</sup> from Tower pond (BOREAS project site, northern Manitoba, Canada) in June 1994 and redissolved at its natural concentration in low organic content deionized water. Bacterioplankton were concentrated from the site by differential filtration<sup>3</sup> and inoculated into irradiated or non-irradiated fulvic acid solutions with no amendments (control), or with amendments of 4.5  $\mu\text{M}$  P as  $\text{PO}_4$  (P treatment); 40  $\mu\text{M}$  N as  $\text{NH}_4\text{NO}_3$  (N treatment); both N and P (NP treatment); 116  $\mu\text{M}$  labile C as glucose (G treatment); or glucose, N and P (GNP treatment). Initial bacterial concentrations averaged  $2.2 \times 10^5$  bacteria  $\text{ml}^{-1}$ . Bacterial growth on fulvic acids was measured as rates of incorporation of  $^3\text{H}$ -leucine into bacterial protein<sup>33</sup> at 0, 20, 52 and 92 h after inoculation;  $n = 3$ ,  $\pm 1$  s.d. Rates of growth were significantly higher in the irradiated treatment in both 52 and 92 h samples in a, b and d ( $P < 0.05$ , Mann-Whitney U-test). Rates of growth were significantly higher in the irradiated treatment in 52 h samples in c and e. There were no significant differences between irradiated and non-irradiated treatments in f.

TABLE 1 Estimated photochemical production of ammonium from aquatic dissolved organic matter

| Sample                                | Production rate ( $\mu\text{M h}^{-1}$ ) | Normalized production rate ( $\mu\text{M m h}^{-1} \times 10^3$ ) | DOC ( $\mu\text{M}$ ) | DON ( $\mu\text{M}$ ) | Fractional conversion ( $\text{h}^{-1} \times 10^3$ ) | pH  | Exposure time (h) | Exposure region | Exposure type |
|---------------------------------------|------------------------------------------|-------------------------------------------------------------------|-----------------------|-----------------------|-------------------------------------------------------|-----|-------------------|-----------------|---------------|
| Boreal pond, whole (July)             | $0.15 \pm 0.010$                         | 3.5                                                               | 3,000                 | 55                    | 2.7                                                   | 7.6 | 18                | Full            | Artificial    |
| Boreal pond, whole (July)             | 0.11                                     |                                                                   | 3,000                 | 55                    |                                                       | 7.6 | 18                | >320 nm         | Artificial    |
| Boreal pond, whole (July)             | 0.080                                    |                                                                   | 3,000                 | 55                    |                                                       | 7.6 | 18                | >360 nm         | Artificial    |
| Boreal pond inlet, fulvic acid (June) | $0.37 \pm 0.010$                         | ND                                                                | 895                   | ND                    |                                                       | 6.6 | 4.8               | Full            | Natural       |
| Boreal pond, fulvic acid (August)     | $0.065 \pm 0.010$                        | 2.5                                                               | 1,133                 | 24                    | 2.7                                                   | 6.5 | 6.6               | Full            | Artificial    |
| Okefenokee swamp, whole               | $0.34 \pm 0.030$                         | 3.2                                                               | 3,840                 | 86                    | 3.4                                                   | 3.9 | 18                | Full            | Artificial    |
| Okefenokee swamp, whole               | 0.22                                     |                                                                   | 3,840                 | 86                    |                                                       | 3.9 | 18                | >320 nm         | Artificial    |
| Okefenokee swamp, whole               | 0.059                                    |                                                                   | 3,840                 | 86                    |                                                       | 3.9 | 18                | >360 nm         | Artificial    |
| Okefenokee swamp, whole               | 0.040                                    |                                                                   | 3,840                 | 86                    |                                                       | 3.9 | 18                | >425 nm         | Artificial    |
| Satilla River estuary, fulvic acid    | $0.050 \pm 0.015$                        | 4.2                                                               | 787                   | 20                    | 2.5                                                   | 5.6 | 8                 | Full            | Natural       |
| Suwannee River, whole                 | 0.36                                     |                                                                   | ND                    | 74                    |                                                       | ND  | 36                | >320 nm         | Artificial    |
| Oyster River, fulvic acid             | 0.32                                     | 3.2                                                               | 2,840                 | 36                    | 9.2                                                   | ND  | 18                | Full            | Artificial    |
| Fluka, humic acid                     | 0.23                                     | 1.8                                                               | 1,942                 | 51                    | 4.7                                                   | ND  | 18                | Full            | Artificial    |

Whole water samples or humic substance fractions were filtered through 0.22  $\mu\text{m}$  membrane filters and stored at 4  $^{\circ}\text{C}$ . Samples were exposed to natural sunlight (33 $^{\circ}$  N, 83 $^{\circ}$  W, Athens, Georgia, USA) or artificial sunlight (DSET Heraeus solar simulator). For some samples, the irradiance was attenuated in the ultraviolet using filters; wavelengths indicated correspond to 50% transmittance. Exposure time is expressed as approximate hours of midday natural sunlight at 33 $^{\circ}$  N latitude in January (860  $\text{W m}^{-2}$  for the 200–3,000 nm wavelength range). Ammonium concentrations were measured by ion chromatography using conductivity detection (Alltech Cation/R column; 3 mM  $\text{HNO}_3$  eluent)<sup>28</sup> or by the Koroleff method<sup>29</sup>. Normalized production rates for samples exposed to full spectrum sunlight were calculated by dividing hourly production rates by each sample's absorption coefficient at 350 nm (ref. 7). DOC, dissolved organic carbon. DON, dissolved organic nitrogen. Fractional conversion rates for samples exposed to full spectrum sunlight were calculated by dividing the hourly production rates of ammonium by the concentration of DON in the sample. DON concentration for the Satilla River estuary sample was estimated assuming a 40:1 C:N ratio for Satilla River fulvic acids<sup>20</sup>. Where standard deviations are indicated,  $n = 2$  or  $3 \pm 1$  s.d. ND, not determined. The boreal pond site is located in northern Manitoba, Canada; the Okefenokee swamp, Satilla River estuary, and Suwannee River sites are in southern Georgia, USA; and the Oyster River site is in New Hampshire, USA. Fluka humic acid is a commercial preparation.



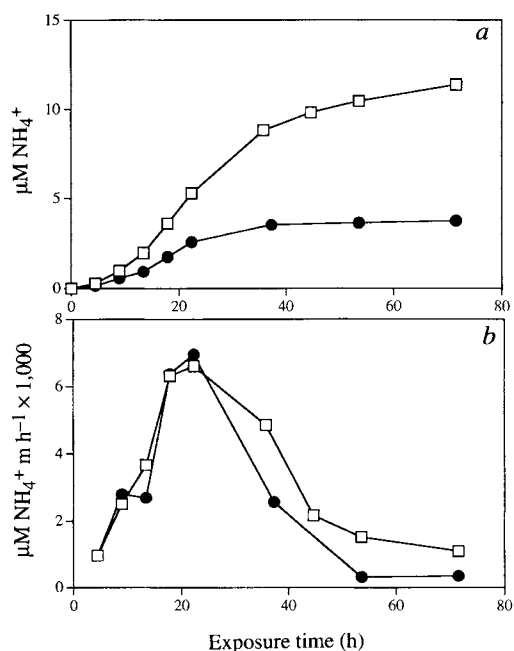


FIG. 2 Concentrations of ammonium (a) and absorptivity-normalized rates of ammonium formation (b) in irradiated natural waters from a boreal pond (northern Manitoba, Canada) (●) and from the Okefenokee swamp (Georgia, USA) (□). Water samples were sealed in quartz tubes with no headspace and were irradiated in a DSET Heraeus solar simulator at  $860 \text{ W m}^{-2}$  (200–3,000 nm wavelength range) for 72 h. Concentrations of ammonium were determined at intervals during continuous irradiation by ion chromatography using conductivity detection<sup>26</sup>. Absorptivity-normalized rates were calculated by dividing average hourly production rates by the average absorptivity of the sample at 350 nm (ref. 7).

#### BOX 1 Coastal photo-ammonification

PHOTOCHEMICAL ammonification from DOM on the southeastern United States continental shelf was calculated using near-surface daily fractional conversion rates of riverine DON to ammonium for Satilla River estuary water in July (this work), modified based on relative action spectra that have previously been determined for DOM photodegradation<sup>6</sup> and average seasonal solar spectral irradiance at  $32^\circ \text{N}$  latitude (assuming a 15% reduction in ultraviolet irradiance from average daily cloud cover)<sup>6</sup>. Seasonal estimates of  $23.8 \times 10^{-3} \text{ d}^{-1}$  (spring),  $26.7 \times 10^{-3} \text{ d}^{-1}$  (summer),  $16.8 \times 10^{-3} \text{ d}^{-1}$  (autumn), and  $12.2 \times 10^{-3} \text{ d}^{-1}$  (winter)<sup>6</sup> were then averaged across seasons and corrected for light attenuation with depth<sup>30,31</sup> to give corrected daily fractional conversion rates of  $0.40 \times 10^{-3} \text{ d}^{-1}$  for the inner shelf (0–20 m depth, 1 m photic zone),  $8.5 \times 10^{-3} \text{ d}^{-1}$  for the mid-shelf (20–40 m depth, 50 m photic zone), and  $5.1 \times 10^{-3} \text{ d}^{-1}$  for the outer shelf (40–60 m depth, 50 m photic zone). The DON available for photochemical conversion in each litre of exported river water was estimated to be 0.21 mg N, calculated from the weighted average of DON in river water in the southeastern United States<sup>20–22</sup> and assuming half the DON pool to be photochemically active. The average residence time of riverine DON on the southeastern United States shelf was taken to be 75 d: 25 d on the inner shelf, 43 d on the mid-shelf and 7 d on the outer shelf<sup>22,31,32</sup>.

From these calculations, 6.0  $\mu\text{mol}$  ammonium are estimated to be produced photochemically from each litre of water exported from rivers to the southeastern United States continental shelf. On an annual basis,  $0.44 \times 10^4$  tons N (as ammonium) are formed on the shelf from exported DON, with  $0.30 \times 10^4$  tons N formed on the mid-shelf alone. Relative to dissolved inorganic nitrogen export, photochemical ammonification increases calculations of available terrestrially derived N in this coastal ecosystem by 20%. □

assimilable nitrogen for bacterial degraders, a finding in conflict with the reported recalcitrance of humic nitrogen. However, nitrogen sufficiency was only observed after exposure to sunlight, indicating that photochemical processes made the humic nitrogen available to natural bacterial populations.

To confirm that assimilable nitrogen was indeed being released photochemically from humic substances, natural DOM and/or humic substances from five environments and a commercial humic acid were exposed to sunlight in quartz vessels. Chemical analysis indicated that ammonium was formed in all samples during exposure to solar radiation (Table 1), regardless of the matrix (that is, unmanipulated DOM or humic substances reconstituted in deionized water); alteration of the solar spectrum by filters demonstrated that ultraviolet wavelengths (295 to 360 nm) were primarily responsible. In long-term irradiations of DOM from two sites with simulated sunlight (equivalent to nine sunny days<sup>7</sup>), kinetics of ammonium formation were found to be complex, with a lag of 22 h before peak formation rate (Fig. 2); these data suggest the possibility of a multistep mechanism for photochemical ammonification. Initial rates of ammonium formation in these two samples were dependent on exposure time but were nearly identical when normalized to the absorbance at 350 nm, a wavelength at which absorbance is approximately proportional to humic substance concentration. During longer exposures, photo-production rates of ammonium were more variable (Fig. 2), as were overall conversions of dissolved organic nitrogen (DON) to ammonium (16% conversion for the Okefenokee swamp, 7% for the boreal pond). The high rate of ammonium production measured for the June boreal pond sample (Table 1) suggests that photochemical ammonification may be greatest for DOM with little prior exposure to sunlight, in this case immediately after the spring thaw.

Element analysis of typical aquatic humic substances indicates that nitrogen accounts for 0.5 to 2% of humic substances by weight<sup>2</sup>. About 25 to 50% of this N appears to be an integral and unreactive component, although relatively little is known about its exact chemical structure<sup>15</sup>. The remainder includes a mixture of amino sugars and other nitrogen-rich compounds that, under acidic conditions, hydrolyse to ammonia and amino acids<sup>2,16</sup>. We propose that this latter class of compounds, although clearly not biologically available when associated with humic substances (Fig. 1), can be converted to ammonium and other assimilable forms of nitrogen by photochemical processes and subsequent hydrolysis reactions. For example, ketones and aldehydes, known constituents of humic substances, photoreact with organic amines to produce imines that hydrolyse to ammonium<sup>17</sup>. Radiolabelled amino acids complexed to soil humic substances have likewise been found to be biologically unavailable until irradiated with ultraviolet light<sup>18</sup>.

The bacterial bioassays indicate that conversion of DON to a readily assimilable form resulted in an increase in the rate of degradation of humic substances. Based on increases in bacterial biomass and assuming a 30% carbon conversion efficiency<sup>3</sup>, we calculate that bacteria used 1.3% of photodegraded boreal pond humic carbon in 52 h when inorganic nitrogen and phosphorus were both limiting, but 2.6% when inorganic nitrogen was the only nutrient in limited supply. Assuming first-order degradation kinetics, the release of assimilable nitrogen results in a decrease in the half-life of photodegraded humic substances from 124 to 57 d under inorganic nitrogen limitation. Nitrogen is generally limiting to production in boreal ecosystems<sup>19</sup>, suggesting that the photochemical release of ammonium and possibly other biologically available nitrogenous compounds from humic substances may be a key process regulating microbial turnover of DOM in these systems.

This discovery of photochemical ammonification of DOM will alter current views of the sources of nitrogen available to fuel autotrophic and heterotrophic processes in planktonic environments. For example, organic nitrogen is a dominant form of terrestrial N exported to the coastal waters of the southeastern

United States ( $2.2 \times 10^4$  tons year<sup>-1</sup>; 48% of total N export<sup>20-22</sup>). Although terrestrially derived organic N has previously been considered unavailable to coastal plankton<sup>23,24</sup>, our calculations indicate that  $\sim 0.44 \times 10^4$  tons of ammonium can be produced photochemically from DOM on the southeastern United States shelf annually (see Box 1). This value increases calculations of the terrestrial N available to microorganisms on the shelf by 20%, and suggests the importance of this and other coastal regions as sites of DON decomposition in the ocean. The minimal light penetration on the inner shelf of the southeastern United States greatly reduces photoreaction rates, and thus most of the photochemical ammonification ( $\sim 70\%$ ) occurs on the mid-shelf, a region not expected to receive significant terrestrial inorganic N through riverine export<sup>24</sup>.

The general importance of photochemical N release from DOM is also suggested by previous studies in Swedish coastal waters<sup>25</sup>, in which additions of riverine humic substances were found to increase nitrogen availability and stimulate rates of primary and secondary production. Worldwide, DON is estimated to account for almost 70% of the N that enters coastal oceans in rivers ( $10 \times 10^{12}$  g N yr<sup>-1</sup>; ref. 26), and future changes in global climate and precipitation patterns are expected to increase the movement of riverine DON to the sea<sup>27</sup>. Ultimately, the participation of DON in autotrophic and heterotrophic biomass production, N<sub>2</sub>O production, denitrification, eutrophication and other fundamental biogeochemical processes requires conversion to biologically available forms. This previously unrecognized mechanism for DON conversion to assimilable N by photochemistry significantly alters current understanding of the sources of biologically active nitrogen in aquatic ecosystems. □

## Determination of olivine cooling rates from metal-cation ordering

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THE mineral olivine—(Fe,Mg,Mn)<sub>2</sub>SiO<sub>4</sub>—is the dominant phase in the Earth's upper mantle, and is also present in a wide range of igneous rocks. Metal cations in olivine crystals are partitioned between two structurally distinct octahedral sites, a property which could in principle be used to obtain important information regarding the thermal history of the host rock. But attempts to establish the temperature and pressure dependence of cation ordering, mainly from the room-temperature structures of samples that have been annealed and quenched<sup>1-3</sup>, have yielded contradictory information. In fact, recent studies have shown that considerable re-ordering occurs during the quenching process<sup>4,5</sup>, and thus cation ordering is unlikely to be representative of high-temperature equilibration. Here we present a new model of the thermodynamics and kinetics of metal partitioning in olivine, derived from *in situ* neutron-diffraction measurements of cation ordering in the synthetic olivine (Fe<sub>0.5</sub>Mn<sub>0.5</sub>)<sub>2</sub>SiO<sub>4</sub>. Our results suggest that the room-temperature structure of a quenched olivine reflects the rate at which the mineral cooled. The extension of this approach to common rock-forming olivines should provide a valuable 'geospeedometer' for determining the cooling rates of rocks that have cooled relatively rapidly.

It has been recognized for some time that non-equilibrium cation ordering in minerals might provide a means to measure rock cooling rates, from early work on Mg/Fe ordering in amphiboles<sup>6</sup> and extensive studies of pyroxenes<sup>7,8</sup> to more recent analysis of Al/Si ordering in alkali feldspars<sup>9</sup>. Displacive phase transitions and exsolution phenomena can obscure the behaviour of these rather complex phases, however, and cation ordering in structurally simple olivine might provide a more robust method of determining thermal histories. The ability to determine cooling rates of olivine phenocrysts from partitioning of the cations over the structurally distinct M1 and M2 octahedral sites would be invaluable in a number of geological scenarios. For example, ancient strombolian-type eruptions could be distinguished from eruptions involving less-rapid quenches on the basis of expected differences in M-site partitioning of olivines contained within them. Such a geospeedometer could also be applied to understanding the cooling processes of high-level alkali olivine basaltic sills, which contain picritic units formed by emplacement of magmas carrying suspended olivine (such as observed in the Scottish Tertiary province). Cooling rates measured from an olivine geospeedometer would resolve the serious disagreement over whether such sills cooled rapidly by a process involving convection<sup>10</sup> or more slowly, mainly by conduction<sup>11,12</sup>. The ability to determine olivine cooling rates would transform our appreciation of a host of such problems in igneous petrology, so far unaddressed because of the lack of a reliable method.

Geospeedometry from intracrystalline cation partitioning demands precise and accurate knowledge of site occupancies. To test the viability of olivine geospeedometry we therefore chose first to focus on Fe–Mn intracrystalline exchange, exploiting the very large contrast between the neutron scattering lengths of Fe

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TABLE 1 Cooling rates of Fe–Mn olivines

| Sample  | Comments                                              | Reference | $X^{\text{Fe}}$ in |       | $X^{\text{Mn}}$ in |       | $Q$   | Cooling rate, $-dT/dt$ ( $\text{K s}^{-1}$ )                   |
|---------|-------------------------------------------------------|-----------|--------------------|-------|--------------------|-------|-------|----------------------------------------------------------------|
|         |                                                       |           | M1                 | M2    | M1                 | M2    |       |                                                                |
| Te40S-1 | Cooled slowly from 1,150 °C                           | 16        | –                  | –     | 0.25               | 0.55  | 0.375 | 0.06                                                           |
| Te40S-2 | Quenched from 950 °C                                  | 16        | –                  | –     | 0.23               | 0.48  | 0.352 | 1.0                                                            |
| Te52.3  | Quenched from 1,000 °C                                | 16        | –                  | –     | 0.35               | 0.70  | 0.333 | 5                                                              |
| OL2     | Synthetic 30 mol% Fa                                  | 17        | 0.404              | 0.197 | –                  | –     | 0.344 | 2                                                              |
| OL3     | Synthetic 47 mol% Fa                                  | 17        | 0.611              | 0.311 | –                  | –     | 0.325 | 10                                                             |
| OL6A    | Natural 55 mol% Fa from amphibolite-grade rock        | 17        | 0.789              | 0.276 | –                  | –     | 0.482 | $\sim 10^{-12}$ below 170 °C (cooled in equilibrium to 170 °C) |
| OL6B    | As OL6A but held at 1,000 °C for 7 days then quenched | 17        | 0.732              | 0.348 | –                  | –     | 0.356 | 0.5                                                            |
| Kn      |                                                       | 18        | 0.661              | 0.367 | 0.294              | 0.633 | 0.326 | 10                                                             |

Using equation (3), the reported room-temperature occupancies of Fe and/or Mn in M1 and M2 sites of synthetic and natural Fe–Mn olivines have been used to calculate cooling rates. Sample names are as given in the original references with compositions expressed as mol% fayalite (Fa =  $\text{Fe}_2\text{SiO}_4$ ) where appropriate. All samples except Te40S-1 and OL6A were synthesized or heat-treated at high temperature and quenched at an unspecified rate. We find a range of quench rates between 0.5 and  $10 \text{ K s}^{-1}$ . Shinno<sup>16</sup> states that Te40S-1 was cooled more slowly: we calculate a cooling rate two orders of magnitude slower than the quench. OL6A, an olivine from a regionally metamorphosed iron formation, appears to have cooled to 170 °C along the equilibrium curve and then taken around  $5 \times 10^6$  years to cool to room temperature.

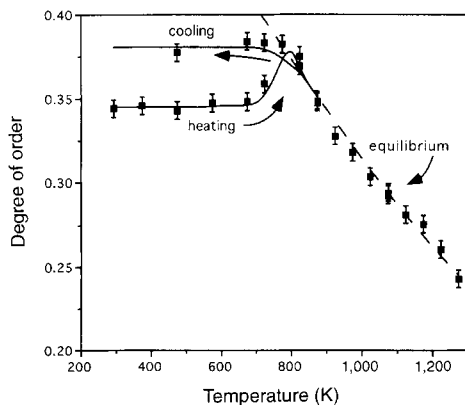


FIG. 1 Heating and cooling behaviour of the degree of order ( $Q$ ) of  $(\text{Fe}_{0.5}\text{Mn}_{0.5})\text{SiO}_4$  olivine. Data points were obtained from the measured site occupancies of the M1 and M2 sites, determined by neutron powder diffraction. Error bars represent  $\pm 1\sigma$ . The dashed line is the least-squares fit of the data above 850 K to the equilibrium curve expected from equation (1). The solid lines show the fit obtained using equation (3) and the known heating and cooling cycles of the experiment. On heating, the non-equilibrium pathway oversteps the equilibrium curve, first ordering towards equilibrium and then disordering onto the equilibrium line. The difference between the values of  $Q$  obtained at room temperature at the start and finish of the experiment reflect the difference in cooling rates between the original quench of the sample from synthesis and the slow cooling of the sample during neutron data collection.

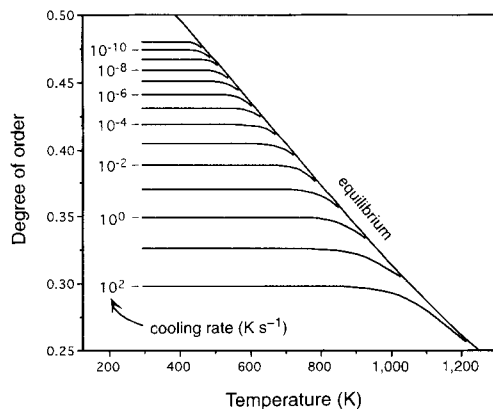


FIG. 2 Cooling paths expected on quenching olivine from the high-temperature equilibrium curve at various cooling rates, calculated from equation (3). The room-temperature value of  $Q$  (M1–M2 partitioning) reflects the rate at which the sample cooled.

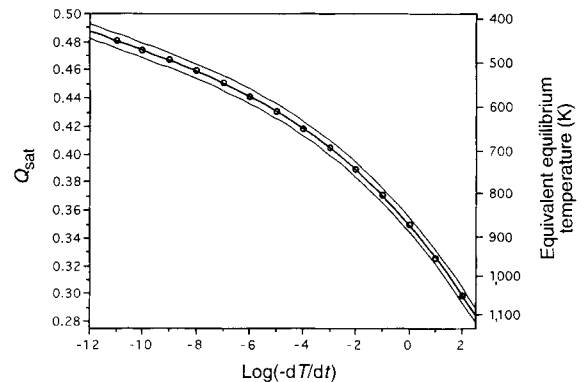


FIG. 3 Dependence of the low-temperature saturation value of M-site order,  $Q_{\text{sat}}$ , on the cooling rate ( $\log$  of rate in  $\text{K s}^{-1}$ ). Circles are room-temperature values of  $Q$  determined from equation (3), the solid line is a second-order polynomial fit through the data. The two outer curves correspond to the error limits of our determination of M-site occupancy by neutron diffraction. The right-hand ordinate gives the temperature corresponding to  $Q_{\text{equilibrium}} = Q_{\text{sat}}$ .

and Mn. High-temperature neutron time-of-flight diffraction patterns of a synthetic  $(\text{Fe}_{0.5}\text{Mn}_{0.5})\text{SiO}_4$  olivine powder were collected at the Polaris diffractometer of the ISIS facility, Rutherford Appleton Laboratory<sup>7</sup>. Full Rietveld structure refinements reveal significant changes in the M-site occupancies on changing temperature (Fig. 1). Small coupled changes in M–O bond lengths and cell edges were observed, and will be elaborated elsewhere together with further details of the refinements.

On heating, at around 400 °C,  $X_{\text{M2}}^{\text{Mn}}$  (the proportion of Mn in M2) begins to increase, until around 600 °C, above which it decreases continuously with increasing temperature. These higher-temperature data represent equilibrium states of order. On cooling, the degree of order saturates below equilibrium order at temperatures less than 500 °C.

We have used these data to develop a thermodynamic and kinetic model for cation ordering in Fe–Mn olivine to test the viability of this method of geospeedometry. Adopting a Landau model for the non-convergent disordering behaviour<sup>13</sup>, the free-energy change due to ordering on M1 and M2 relative to a fully disordered state may be expanded in the order parameter  $Q$  as:

$$\Delta G = -hQ + \frac{a}{2}(T - T_c)Q^2 + \frac{b}{4}Q^4 \quad (1)$$

where  $h$ ,  $a$ ,  $b$  and  $T_c$  are material-dependent parameters. For our experiment  $Q$  is defined as  $(X_{\text{M1}}^{\text{Mn}} - X_{\text{M2}}^{\text{Mn}})/(X_{\text{M1}}^{\text{Mn}} + X_{\text{M2}}^{\text{Mn}})$  (that is,



$Q = 1$  for complete order, and 0 for total disorder). The equilibrium temperature-dependence of  $Q$  is given by the condition  $\partial(\Delta G)/\partial Q = 0$ . Fitting the data to this condition yields  $a/h = 0.00523$ ,  $b/h = 13.4$  and  $T_c = 645$  K. The lower-temperature kinetically controlled  $Q$ - $T$ -time( $t$ ) pathways are defined by the Ginzburg-Landau equation<sup>14</sup>:

$$\frac{dQ}{dt} = -\frac{\gamma \exp(-\Delta G^*/RT)}{2RT} \frac{\partial \Delta G}{\partial Q} \quad (2)$$

where  $\gamma$  is a frequency factor and  $\Delta G^*$  is the activation energy for M1-M2 exchange. Substituting equation (1) into equation (2) and integrating we obtain:

$$t - t_0 = \int_{Q_0}^Q \frac{-2RT}{\gamma h \exp(-\Delta G^*/RT) \left( -1 + \frac{a}{h}(T - T_c)Q + \frac{b}{h}Q^3 \right)} dQ \quad (3)$$

which we solve numerically to obtain the time-dependence of ordering behaviour away from equilibrium on both heating and cooling. The neutron data have been fitted to this solution, adopting the known  $T$ - $t$  path of the experiment. The fit is shown in Fig. 1 by solid curves (non-equilibrium heating and cooling), and gives an activation energy of Mn-Fe exchange on M1 and M2 of  $193 \pm 3$  kJ mol<sup>-1</sup>. In doing so we have assumed  $\log(\gamma h) = 13.1 \pm 0.5$ , following the arguments of Harrison and Putnis in their analysis of cation ordering in spinel<sup>15</sup>, as no calorimetric data exists for the enthalpy of ordering in olivine (from which one may compute  $h$ ). Changing the activation energy changes the temperature at which the 'peak' of the  $Q(T)$  curve occurs on heating.

Using these values, we can simulate ordering paths expected at any cooling rate. On rapid cooling,  $Q$  falls below the equilibrium path at some temperature  $T'$  and a low value is frozen-in down to low temperatures. Slower cooling generates higher values of 'quenched-in'  $Q$  at room temperature. We refer to this as the saturation value of  $Q$ ,  $Q_{\text{sat}}$ , which increases with slower cooling rates. The M-site occupancies measured at room temperature thus reflect the cooling rate of a quench from temperatures above  $T'$ . This reiterates the conclusion that published quench experiments on olivine do not describe the equilibrium high-temperature partitioning behaviour, but instead simply reflect particular cooling of individual experiments. Furthermore, this indicates that the room-temperature structure of an olivine may be used as a geospeedometer for rapid cooling events, such as in volcanic rocks. The range of cooling rates is suggested by Fig. 2, which shows the expected  $Q$ - $T$  paths of  $(\text{Fe}_{0.5}\text{Mn}_{0.5})_2\text{SiO}_4$  olivine cooled at constant rates over 13 decades of  $dT/dt$ . Adopting this method we find that the initial value of  $Q$  of our  $(\text{Fe}_{0.5}\text{Mn}_{0.5})_2\text{SiO}_4$  sample corresponds to a quench rate (from its initial synthesis conditions) of  $1.5 \pm 0.2$  K s<sup>-1</sup>, which agrees with our estimate of the average quench rate of the sample from the synthesis temperature. Equation (3) could equally easily be used to determine  $Q$ - $T$  paths following Newton's law of cooling, or other more elaborate time-temperature paths such as the step cooling path of our neutron diffraction experiment.

By calculating the  $Q$ - $T$ - $t$  paths across a range of cooling rates, the relationship between  $dT/dt$  and  $Q_{\text{sat}}$  has been obtained (Fig. 3). We find a nonlinear relationship between  $Q_{\text{sat}}$  and  $\log(dT/dt)$ . The temperature corresponding to an equilibrium value of  $Q = Q_{\text{sat}}$  is given on the right-hand ordinate. Our neutron experiments provided site occupancies with an error of less than 1%. Assuming that the same procedure can be carried out for Fe-Mg olivines crystallizing from picritic and basaltic liquids, and that site occupancies can be determined to 10%, this implies that at cooling rates slower than around  $10^{-5}$  K s<sup>-1</sup> the changes in cation partitioning would be less than the limits of precision, and hence it would be more difficult to resolve cooling rates slower than around 1 K per day. As well as being applied to deducing the

eruption/cooling rates of volcanic rocks, we expect that it should be possible to obtain information on the cooling of high-crustal level, relatively thin, sheet-like bodies of thickness 10–15 m, and on the cooling of the outer parts of intrusions substantially larger than this, of the order of 50–100 m thick.

These results show that the cooling rate of an Fe-Mn olivine may be determined from its room-temperature structure alone, specifically from the M1-M2 site occupancies. We further illustrate this in Table 1, where we have used the reported room-temperature M1-M2 site occupancies of a number of natural and synthetic Fe-Mn olivines to determine their cooling rates. Non-convergent ordering in olivines clearly provides a viable new method for high-speed geospeedometry. We are pursuing this approach and its application in planned and current studies of Fe-Mg, Fe-Ni and Mg-Mn ordering in olivines. □

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## Constraints from seismic anisotropy on the nature of the lowermost mantle

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**THE D'' layer lies at the bottom of the Earth's rocky mantle, and separates it from the liquid metal-alloy core. This region, extending from the core-mantle boundary to a few hundred kilometres above (Fig. 1), is geodynamically analogous to the more easily studied lithosphere, at the top of the mantle. The structure of D'' may reflect the style of lower-mantle convection, the nature of core-mantle interaction and perhaps even the fate of subducting lithosphere<sup>1</sup>. Observations of lithospheric seismic anisotropy have provided valuable insight into the nature of the upper-mantle boundary layer, but discussion of lower-mantle seismic anisotropy has been somewhat contentious<sup>2–5</sup>. Here we present evidence, from seismic waves that have traversed the lowermost mantle beneath the Caribbean region, for a zone of seismic anisotropy below the D'' discontinuity, which in this region lies 250 km above the core-mantle boundary. The anisotropy is most probably due to horizontal layering or aligned inclusions of a material with differing shear-wave velocity. If D'' is a graveyard for subducted lithosphere, a plausible explanation of the anisotropy may be the contrast between cold lithospheric mantle and material that formerly constituted the oceanic crust, which may have lower shear-wave velocity owing to the presence of melt.**

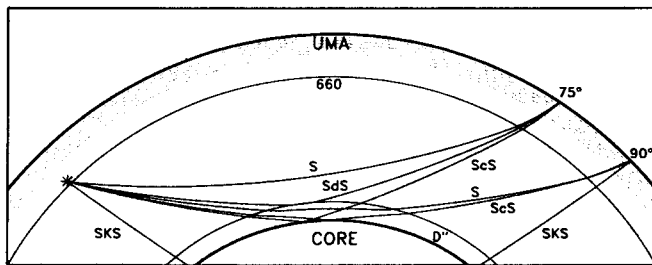


FIG. 1 A sketch of the seismic S-wave phases analysed at epicentral distances 75° and 90°. The 660-km discontinuity separates the upper and lower mantle; UMA refers to the region of upper-mantle anisotropy. Like those analysed in this study, the earthquake (indicated by an asterisk) is near the bottom of the upper mantle. In our study area a seismic discontinuity lies atop the D'' region 250 km above the core-mantle boundary<sup>14</sup>.

Seismic tomography has shown that the largest lateral variations in lower-mantle velocities are at the base of the mantle<sup>6,7</sup>. Similarly, studies of core-diffracted phases have shown strong velocity variations within D'' (ref. 8). Lay and Helmberger<sup>9</sup> presented the first definitive evidence for a D'' discontinuity 250–300 km above the core-mantle boundary (CMB); later analyses of P- and S-wave data have revealed that the discontinuity exhibits

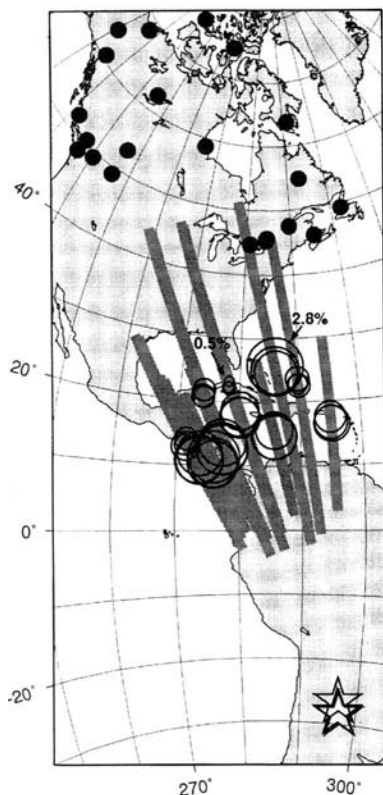


FIG. 2 A Cassini equal-area projection of the region of study. Filled circles show the stations of the Canadian National Seismograph Network (CNSN), and the stars show the epicentres of the three earthquakes studied. The range of epicentral distances spanned by the network is 70° to 110°. The interpreted S-wave anisotropy is plotted at the surface projection of source/station midpoints and the size of the circle is proportional to the degree of anisotropy (the extreme cases are marked). The extent of the sampled D'' region is indicated by a grey swath for each event-station raypath which enters D''

strong lateral variations and is not a global feature<sup>9–13</sup>. It has also been proposed that D'' may be anisotropic<sup>2,4</sup>. Observations of anomalous high-amplitude diffracted SV phases (shear waves polarized in the source-receiver plane and diffracted along the CMB) have been attributed to D'' anisotropy, but these observations have been somewhat inconclusive as they can be explained with relatively simple isotropic structures<sup>5</sup>. Here we present strong evidence for D'' anisotropy in a region below Central America and the Caribbean (Fig. 2). Unlike previous studies, our analysis uses broad-band data and concentrates on travel times for S-wave arrivals which are primarily pre-diffracted. Our data set is unique in that we have sufficient coverage to analyse phases which turn above and within the D'' region thereby allowing us to constrain the anisotropy to the D'' region.

The broad-band data are from South American earthquakes recorded on the recently refurbished Canadian National Seismograph Network (CNSN; Fig. 2). Horizontally polarized, shear-wave (SH) data that image this region show clear evidence of a precursor to the core-reflected phase ScS. This has been interpreted as a reflection from a discontinuity 250 km above the CMB with a velocity contrast of 2.75% across the interface<sup>9,14</sup>. Here we compare the SH signals with the SV signals, looking for evidence of shear-wave splitting that is diagnostic of seismic anisotropy<sup>15</sup>. The phases we consider are primarily pre-diffracted phases, S and ScS, although five stations record diffracted arrivals, S<sub>diff</sub>.

The earthquakes considered are very deep (>575 km) thus mitigating effects due to source-side upper-mantle anisotropy. The effects of receiver-side upper-mantle anisotropy cannot be dismissed so easily. Waveforms from many Canadian stations exhibit significant amounts of SKS splitting indicating receiver-side upper-mantle anisotropy<sup>15,16</sup>. It is therefore imperative that interpretations of lower-mantle anisotropy must first account for the upper-mantle anisotropy. To do this, we take independent estimates of upper-mantle anisotropy from observations of SKS splitting from events with a wide range of azimuths<sup>15,17</sup>. Measurements of upper-mantle anisotropy have been published for some of the CNSN stations<sup>16</sup> and we have made our own measurements for those stations without published values. Because the SKS measurements are from a variety of azimuths, the observed splitting must be due to anisotropy where ray paths are most similar which, for this case, is the upper mantle. Furthermore, the significant variation in splitting magnitude and orientation between receivers suggests that the anisotropy is confined to the upper mantle<sup>15</sup>.

We localize the source of lower-mantle anisotropy to the D'' region through the analysis of phases that turn above and below the D'' discontinuity. Figure 1 shows the phases under consideration: at 75°, S turns above the D'' region, SdS reflects off the D'' discontinuity (250 km above the CMB in this area) and ScS reflects off the CMB. The splitting measured on S-arrivals that turn in the lower mantle above the D'' region can be entirely explained by anisotropy in the upper mantle as they show good agreement with independent SKS-splitting measurements. It is difficult to measure ScS splitting at these distances (<80°) owing to interference from SKS and SKKS, but the few reliable estimates that we can make show significantly more splitting. In such cases the magnitude and orientation of the ScS splitting is significantly different from that measured for SKS from a range of azimuths at the same station.

Beyond 80° the S and ScS arrivals are only separated by a few seconds in travel time, and are thus hard to isolate in a seismogram. Furthermore, standard splitting analysis is difficult as the radial and transverse component waveforms are quite different (Fig. 3). Instead we simply measure  $\delta t$  from the separation in travel-time onset after making a correction for upper-mantle anisotropy. Whereas the separation in SH and SV travel times for rays which turn above D'' can be explained by upper-mantle anisotropy, those for rays which turn within D'' show greater values of  $\delta t$  thereby constraining most of the lower-mantle anisotropy to the D'' region. The S-ScS and S<sub>diff</sub> data show large delay

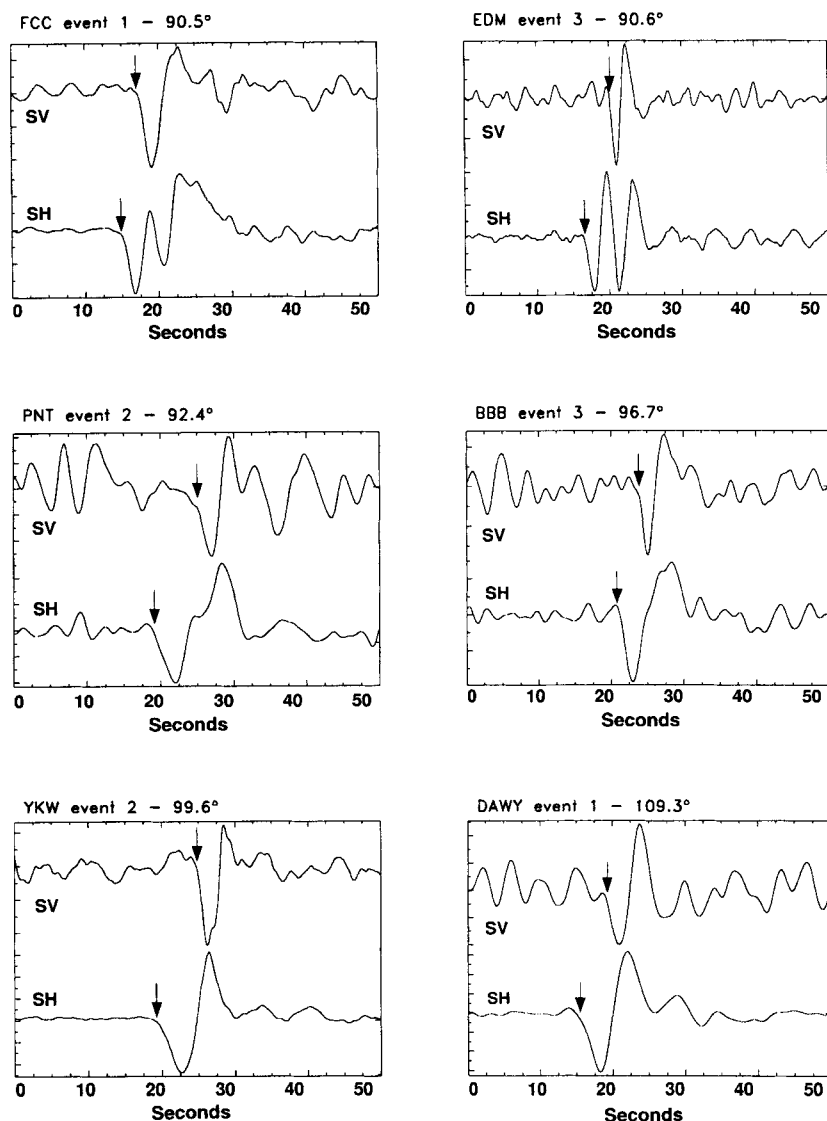


FIG. 3 Travel-time separations between arrivals on the radial (upper) and transverse (lower) components at stations beyond  $90^\circ$ . These seismograms (velocity records) have been corrected for upper-mantle anisotropy and represent  $\sim 15\%$  of the data analysed; they were selected to show the splitting most clearly. The nearer stations record S and ScS arrivals which are only a few seconds apart, while the more distant station, DAWY, records the core diffracted phase,  $S_{\text{diff}}$ . Despite differences in waveform character, it is very clear that there are dramatic differences in the onset of the radial and transverse arrivals with the SH component always leading the SV component.

times (3–9 seconds) with the SH component always leading the SV component (Fig. 3).

The delay time depends both on the intrinsic anisotropy and horizontal extent of the anisotropy, which is in turn also dependent on the assumed thickness of the anisotropic region. As a conservative estimate, we assume that the anisotropic region extends from the CMB to the discontinuity 250 km above the CMB<sup>14</sup> and that the anisotropy is uniformly distributed along the entire path through a  $D''$  model (SKNA1<sup>14</sup>) for this region. The results suggest 0.5–2.8% anisotropy along corridors 1,000–2,800 km in length, with an average values of 1.8% (Fig. 2). Signals that arrive at roughly  $90^\circ$  sample only the upper few tens of kilometres of  $D''$  and yet they still exhibit significant shear-wave splitting. This implies that the anisotropy is vertically distributed throughout most of the  $D''$  region. The variability in the estimated anisotropy may be due to variability in the thickness of the  $D''$  layer throughout this region.<sup>14</sup>

Three important pieces of evidence strongly suggest that  $D''$  beneath the Caribbean is transversely isotropic. First, in our study area the SH phase is always faster than the SV phase. Second, rays with near-vertical paths through  $D''$  (such as SKS) show no sign of splitting from this depth range<sup>18</sup>. This is consistent with transverse isotropy, but not with azimuthal anisotropy. The final argument comes from waveform modelling. The observed waveforms agree well with synthetic reflectivity waveforms for isotropic models with

a discontinuity 250 km above the CMB, except that there is a large travel-time separation between the radial and transverse component seismograms that the isotropic models cannot explain. Such simplicity in the waveforms strongly suggests transverse isotropy because the SH and SV systems are decoupled for this case<sup>19</sup>.

Any model for  $D''$  must satisfy the observed symmetry and magnitude of the anisotropy, and the discontinuous increase in SH-wave velocity ( $v_{\text{SH}}$ ) at the top of  $D''$  in this region<sup>14</sup>. It appears unlikely that the anisotropy is due to the lattice preferred orientation (LPO) of lower-mantle minerals. Recent experiments suggest that perovskite, probably the most abundant mineral in the lower mantle, fails to develop strain-induced LPO<sup>18</sup>. It is also difficult to satisfy the seismic constraints with the alignment of other lower-mantle minerals with significant single crystal anisotropy (for example, cubic MgO and tetragonal stishovite). It is more probable that the anisotropy within  $D''$  is due to the horizontal alignment of inclusions (within a matrix) or near-horizontal layering of materials with differing isotropic shear velocities<sup>20</sup>. Both models give rise to long-wavelength transverse isotropy with a vertical symmetry axis and exhibit  $v_{\text{SH}} > v_{\text{SV}}$  (where  $v_{\text{SV}}$  is the SV wave velocity) for horizontally propagating waves<sup>21–23</sup>. These models are thus consistent with the form of the observed anisotropy.

We model the anisotropy using a theory for aligned spheroidal inclusions<sup>22,23</sup> (Fig. 4). The magnitude of anisotropy depends



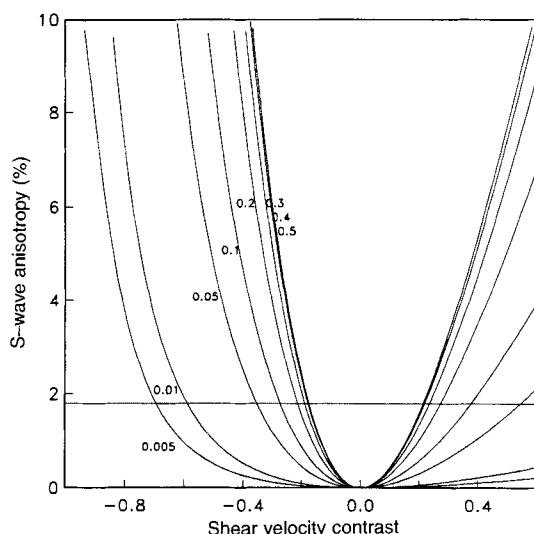


FIG. 4 Modelling the per cent anisotropy due to the preferential alignment of horizontal penny-shaped inclusions using the theory of Tandon and Weng<sup>22</sup> and Sayers<sup>23</sup>. The matrix velocity is  $7.5 \text{ km s}^{-1}$  and the inclusions have an aspect ratio of 0.01. The different curves are the per cent anisotropy as a function of velocity contrast for different inclusion volume-fractions (given on the curves). The shear-velocity contrast is defined as  $(v_2 - v_1)/v_1$ , where  $v_2$  is the velocity of the inclusion material and  $v_1$  is the velocity of the matrix. The average observed anisotropy (1.8%) is marked for reference (horizontal line). As the S-wave velocity of the inclusions tends to zero, very small inclusion volume-fractions can explain the observed anisotropy. Numerical experiments show little variation in S-wave anisotropy as the inclusion aspect-ratio gets smaller (eventually the medium can be effectively treated as being layered). Cigar-shaped inclusions (aspect ratio  $>1.0$ ) require much higher volume fractions to explain the observed anisotropy.

primarily on the volume fraction of the inclusions, the inclusion aspect ratio and the shear velocity contrast. The aspect ratio of the inclusions can vary from that of cigar-shapes to that of penny-shapes, but it is much easier, in terms of orientation and volume fraction, to satisfy the observations with penny-shaped inclusions. As the inclusions get very flat (aspect ratio  $<0.001$ ) there is good agreement with the results for alternating layers<sup>21</sup>.

One simple model that is not consistent with the data has the matrix velocity equal to that of the zone just above  $D''$ , and the inclusion velocity faster. This requires unrealistic inclusion velocities (in excess of  $10 \text{ km s}^{-1}$ ) and predicts too large a velocity discontinuity. This leads to the significant conclusion that both components have elastic properties distinct from the mantle just above  $D''$ . One important class of models that is efficient in

producing anisotropy is characterized by inclusions of partial melt, as its shear velocity is nearly zero. The potential existence of melt in the lower mantle is uncertain, as is the geometry and alignment of such melt inclusions. There is little consensus on the geometry of melt inclusions in even lithospheric rocks<sup>24</sup>, but melt inclusions with aspect ratios of the order of 0.05 have been observed in peridotites<sup>25</sup>. Less than 1% melt can explain the observed anisotropy if we assume that the melt exists in thin ellipsoidal disks (for aspect ratios  $<0.05$ ) that are aligned owing to higher shear stresses. In such small quantities this produces less than a 1% reduction in  $v_{SH}$  and, consequently, the matrix must be about 3–4% fast.

We consider two possible mechanisms for producing a layered  $D''$  region with the appropriate seismological properties. The first is the 'reaction-zone' hypothesis<sup>26,27</sup>, where  $D''$  is thought to contain silicate and iron-alloy products of a reaction between the mantle and core. As iron alloys have shear velocities 30–40% lower than silicates<sup>27</sup>, this model could account for the anisotropy, if a 5% iron-alloy concentration (Fig. 4) segregated into horizontally elongated inclusions, throughout the 250 km thickness of  $D''$ . The introduction of Fe into  $D''$ , however, will have a general tendency to reduce shear velocities. In particular, taking the chemical reaction proposed by Knittle and Jeanloz<sup>26</sup>, the aggregate  $v_{SH}$  of the reaction products is lowered by up to 6% (depending on the amount of Fe reacting), compared to the starting perovskite material.

The second mechanism is motivated by the increasingly compelling seismological observations that slabs descend into the lower mantle<sup>28–30</sup>. In such a scenario, the folded slabs may lie horizontally on the CMB<sup>30,31,32</sup>. The differing seismic properties of the former-harzburgite and former-basalt components of the slab could give rise to the anisotropy, while the high  $v_{SH}$  for  $D''$  could be achieved through the retained thermal anomaly of the slab. Assuming that the basalt fraction is 0.1, then the contrast has to be at least 20% (Fig. 4). If there is a low-melting-temperature component within former-basalt, resulting from the relatively high concentrations of Fe, Al and Ca, a fraction of the former-basalt could have a significantly reduced shear modulus. A simple model is that melt within the basalt lowers its overall seismic velocity, and that the anisotropy results from the layering of the slower basalt and fast lithosphere. Recent melting experiments on probable basaltic components indeed suggest significant lowering (by  $\sim 2,000 \text{ K}$ ) of the melting point of perovskite due to Al and Ca<sup>33</sup>. If true, then 'old slabs' would provide an explanation for the overall properties of this region. Furthermore, lower-mantle tomography shows evidence for a vertical, slab-like feature extending down to the CMB (the so-called Caribbean anomaly), which has been attributed to the subduction of the former Farallon plate<sup>30,34</sup>. The hypothesis of a slab graveyard at the CMB is in principle straightforward to test and if true, would suggest that slabs play a major role in the mantle's two major thermal boundary layers, at the surface and CMB. □

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# Lévy flight search patterns of wandering albatrosses

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**LÉVY flights are a special class of random walks whose step lengths are not constant but rather are chosen from a probability distribution with a power-law tail. Realizations of Lévy flights in physical phenomena are very diverse, examples including fluid dynamics, dynamical systems, and micelles<sup>1,2</sup>. This diversity raises the possibility that Lévy flights may be found in biological systems. A decade ago, it was proposed that Lévy flights may be observed in the behaviour of foraging ants<sup>3</sup>. Recently, it was argued that *Drosophila* might perform Lévy flights<sup>4</sup>, but the hypothesis that foraging animals in natural environments perform Lévy flights has not been tested. Here we study the foraging behaviour of the wandering albatross *Diomedea exulans*, and find a power-law distribution of flight-time intervals. We interpret our finding of temporal scale invariance in terms of a scale-invariant spatial distribution of food on the ocean surface. Finally, we examine the significance of our finding in relation to the basis of scale-invariant phenomena observed in biological systems.**

We collected data over a three-month summer period as part of a study of the foraging biology of albatrosses in the South Atlantic<sup>5</sup>. Electronic recording devices were attached to the legs of five different adult albatrosses on Bird Island, South Georgia (54° 00' S, 38° 06' W), on 19 separate foraging trips. The devices took measurements every 3 s, and recorded  $u(t)$ , the number of 15-s intervals in each hour  $t$  for which the animal was wet for 9 s or more. Each entry in the time series  $u(t)$  is therefore a number from 0 to 240, and  $t = 1, 2, \dots, t_{\max}$  is time measured in hours. We thereby obtain 19 time series, one for each of the foraging trips studied. These range in duration from  $t_{\max} = 77$  h to  $t_{\max} = 416$  h with a mean of 175 h. The birds do not forage on land, and the wet periods indicate interruptions in their flight paths caused by their alighting on water to eat or rest. As an example of the raw data, see Fig. 1.

First, we analysed the data using 'random walk' methods<sup>6</sup>, which can detect the presence of long-range correlations. The net displacement  $y(t)$  of the time series  $u(t)$  is defined by the running sum  $y(t) \equiv \sum_{i=1}^t u(i)$ . An important statistical quantity characterizing the walk is the root mean square (r.m.s.) fluctuation of the displacement,  $F(t) \equiv \sqrt{\langle (\Delta y(t))^2 \rangle - \langle \Delta y(t) \rangle^2}$ , where  $\Delta y(t) \equiv y(t_0 + t) - y(t_0)$ . The angular brackets denote expectation values, that is, averaging over all possible times  $t_0$ . The calculation of  $F(t)$  can distinguish three types of behaviour<sup>7</sup>. Uncorrelated time series give rise to uncorrelated random walks described by

$$F(t) \sim t^{\frac{1}{2}} \quad (1)$$

with  $\alpha = \frac{1}{2}$ , as expected from the central limit theorem. Markov processes also give  $\alpha = \frac{1}{2}$  for sufficiently large  $t$ . In the presence of long-range correlations with no characteristic time scale, equation (1) holds, but with  $\alpha \neq \frac{1}{2}$ .

Figure 2a shows that long-range correlations exist, because we have an almost perfect power law with  $\alpha = 0.84 \pm 0.2 \neq \frac{1}{2}$ . (The error is found by estimating  $\alpha$  for each time series separately.) When we randomly shuffle the data, the long-range correlations vanish and we recover  $\alpha = \frac{1}{2}$  as expected. The power spectrum<sup>8</sup> shown in Fig. 2b,c and detrended fluctuation analysis

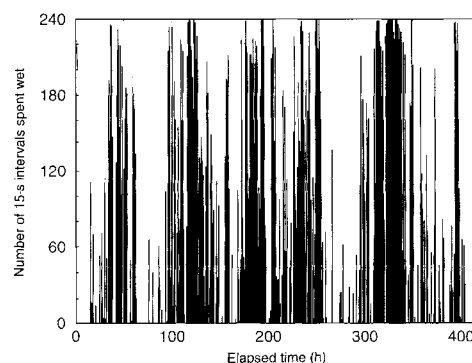


FIG. 1 The longest of the 19 time series, with a length of 416 h. Each point in the time series gives the number of 15-s intervals in each hour for which the animal was wet for 9 s or more.

(DFA)<sup>9,10</sup> confirm the existence of long-range correlations and scale invariance.

Having established the existence of scale invariance, we now turn to the question of its origin. Although scale invariance is widely observed in biology<sup>2,10–18</sup>, the basis for such scale-invariant behaviour has remained elusive. Scale invariance in complex systems could be caused by nonlinear dynamics, as it is well known that nonlinear dynamics can give rise to intermittency, chaos and scale invariance<sup>19</sup>. However, it has been speculated that scale invariance may confer biological advantages related to adaptability of response; for example, loss of scale invariance for heartbeat intervals corresponds to a diseased state<sup>2,12</sup>. Scale invariance in foraging patterns may reflect the exploitation of highly complex environments which might themselves have fractal properties.<sup>20–22</sup>

To study the origin of scale invariance found in our data, we plotted the distribution of flight-time intervals (Fig. 3a), and found for this distribution approximately a power law. To understand this finding, we developed a one-parameter Lévy flight model of bird foraging in which the probability of flying for a time  $t_i$  between landings is

$$P(t_i) \sim (t_i + 1)^{-\mu} \quad (2)$$

where the '+1 term' arises because the bird spends exactly one unit of time on water for each landing. A power-law distribution of flight-time intervals corresponds to a Lévy walk, not a Lévy flight, but the landing points of the bird correspond to points visited in a Lévy flight (see refs 2,23). We observed a value of  $\mu \approx 2$  in the histogram of flight times, so we fixed  $\mu = 2$  in our model, and used simulations to find the r.m.s. fluctuation  $F(t)$ , shown in Fig. 3b. The model predicts long-range power-law correlations with  $\alpha = 0.8 \pm 0.05$ , consistent with the value  $\alpha = 0.84 \pm 0.2$  obtained from the data. (It can be shown for the model that  $\alpha$  approaches the limit  $2 - \mu/2$  asymptotically for sufficiently long sequences.)

Conventional random walks have been used to model the foraging behaviour of bacteria and other organisms<sup>24,25</sup>. However, such models predict not a scale-invariant power law but rather a Poisson distribution, and so cannot explain our experimental findings of temporal scale invariance and Lévy flight behaviour<sup>2,23</sup>. The results of the present quantitative analysis are in conformity with existing qualitative suggestions that this species of seabird specializes in long journeys of 'random foraging'<sup>26</sup>, searching for patchily and unpredictably dispersed prey over several million square kilometres. Interruptions to long foraging flight paths, which occur when the birds forage actively on a restricted oceanic area, could conceivably be related to the presence of ships<sup>26</sup>. As there are few ships in this region of the South Atlantic, we are confident that the effects of ships on our data were minimal during the period of our study.

We wondered why the flight times of birds follow a scale-invariant power-law distribution. We next relate the observed

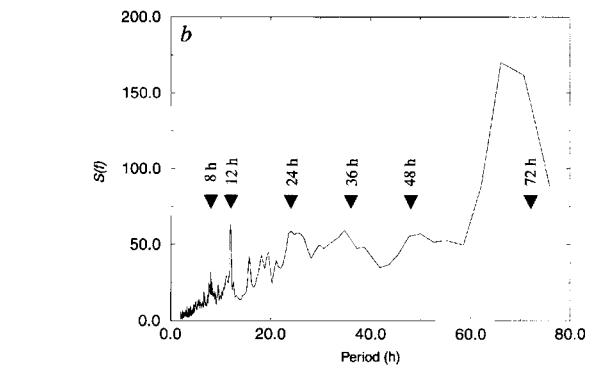
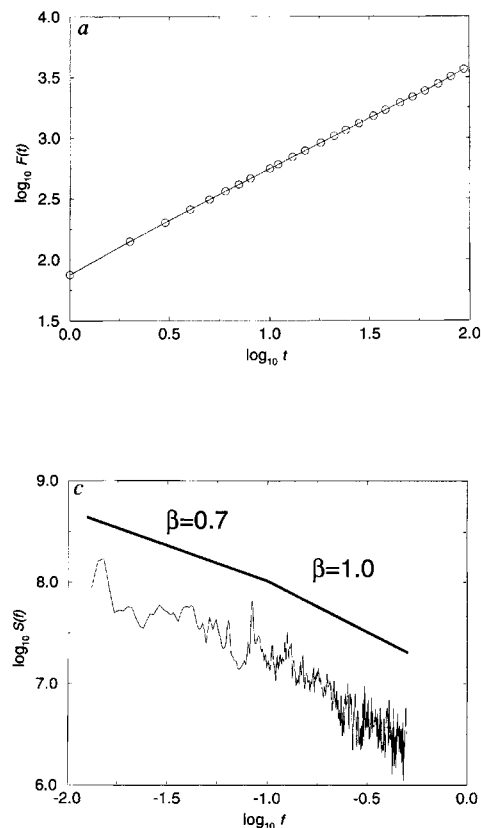


FIG. 2 **a**, Double log plot of the r.m.s. fluctuation  $F(t)$ . The regression fit gives  $\alpha = 0.844 \pm 0.002$  ( $F = 159,000$ ). As  $\alpha > 0.5$ , we conclude that long-range power-law correlations exist in the data. We calculate  $F(t)$  by weighting equally all possible configurations of a moving window of length  $t$  over the 19 time series. **b**, The sum of the power spectra  $S(f)$  of each of the 19 time series in units of  $10^6$ , plotted against the period  $1/f$ . We find 12-h and 24-h components in the spectrum, consistent with the observation that these birds fly more during daytime than at night<sup>5</sup>. We use only those frequencies corresponding to periods smaller than the length of the smallest time series. That is,  $f^{-1} < 77$  h. **c**, Double log plot of the sum of the spectra against frequency  $f$  using logarithmic binning. We define the scaling exponent  $\beta$  by  $S(f) \sim f^{-\beta}$ . The straight lines correspond to  $\beta = 1.0$  and  $\beta = 0.7$ , respectively, showing that the spectrum approximately follows power laws in the two regimes indicated. As  $2\alpha = 1 + \beta$  (ref. 7), the low-frequency (large time scale) value  $\beta \approx 0.7$  is consistent with the value of  $\alpha = 0.84$ . In both regimes, the spectrum is not like the white noise ( $\beta = 0$ ) associated with temporally uncorrelated behaviour, but more like the '1/f' spectra associated with long-range correlations in scale-invariant systems.

temporal behaviour to a possible spatial scale-invariance property in the underlying ecosystem<sup>20</sup>. It is known that the points visited by a Lévy flight form a fractal with dimension  $D = \mu - 1$  (refs 2, 23). Figure 4 illustrates typical flight patterns constructed from the data and from the model, assuming that the distance travelled is proportional to the time spent dry, and that the flight directions change randomly after spending time in water. Although the latter assumption is unrealistic, it is nevertheless equally unrealistic for both the model and for the real data. The landing points of the birds have spatially scale-invariant properties, which may indicate that the distribution of food on the ocean surface is also scale invariant<sup>27</sup>. If this is so, then there would be voids on all length scales where there is little or no food, and birds that fail to produce a scale-invariant distribution of flight-time intervals would face a greater difficulty finding food, and hence surviving. It is also not inconceivable that the power-law distribution of flight intervals may be related to the lifetime distribution of the thermals used by the birds to fly<sup>1</sup>.

An additional potential advantage of Lévy flights, suggested first for insects (M. F. Shlesinger, personal communication), relates to foragers that operate in swarms or flocks. After  $t$  steps, a single brownian walker in two dimensions visits  $t/\ln t$  new sites, whereas a single Lévy walker visits  $t$  new sites<sup>28</sup>. This is not a large difference: Lévy flights are not much better than brownian motion in terms of reaching new sites. But for a 'swarm' comprised of  $N$  walkers, there is a large difference between the brownian walk and the Lévy flight: after  $t$  steps, a brownian swarm of  $N$  walkers visits only  $t \ln(N/\ln t)$  distinct sites until an astronomically large crossover time  $t_x \sim e^N$  is reached<sup>28</sup>, whereas the swarm of Lévy walkers visits  $Nt$  sites. Thus the Lévy flight pattern allows the individual to visit new sites that the swarm has not visited. As some prey can migrate vertically in response to predators, the food at a given site may become unavailable for some time, thereby forcing the birds to find new sites not yet visited. Thus Lévy flight search patterns in animal behaviour may reflect the solution of the biological search problem in complex environments, and it would be interesting to repeat this type of study for birds whose

FIG. 3 **a**, Distribution giving the number  $n_i$  of intervals in the entire data set with flight-time intervals  $t_i$ . We used bin widths of  $2^k$  h for the bin  $k$ , and used the geometric midpoints of the bins to plot the results. The Lévy-walk model of foraging fits the data quite well, as can be seen by the agreement of the data with the straight line of slope  $-2$ . **b**, Double log plot of the r.m.s. fluctuation  $F(t)$  for the model, which gives rise to long-range power-law correlations with  $\alpha > 0.5$ . Also, the numerical value of  $\alpha$  is consistent with the experimental results (Fig. 2a).



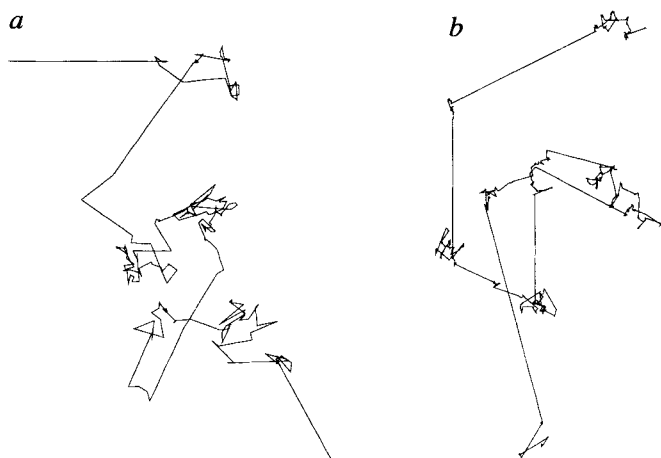


FIG. 4 *a*, Possible flight path of a bird constructed from the longest time series, as described in the text. The time resolution of the data prevents us from considering changes in flight directions which occur more frequently than once per hour. *b*, Possible flight path given by the Lévy-walk model discussed in the text. Both flight paths have scale-invariant 'fractal' properties which may indicate that the distribution of food on the ocean surface is spatially scale invariant.

foraging strategies are different from those of the wandering albatross.

Our findings represent a first attempt at studying Lévy flight search patterns in animal behaviour, and we expect the quality of such studies to improve with better data. So far, only qualitative analyses of data from satellite tracking of the wandering albatross have been presented<sup>26,29,30</sup>. A preliminary analysis of foraging patterns of other species suggests that Lévy flights may be more widespread in nature (see data shown in Fig. 4 of ref. 31). As more data becomes available, quantitative analyses should provide an improved view of the foraging strategies of animals in complex environments. □

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## Sensitivity to leptin and susceptibility to seizures of mice lacking neuropeptide Y

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NEUROPEPTIDE Y (NPY), a 36-amino-acid transmitter distributed throughout the nervous system<sup>1,2</sup>, is thought to function as a central stimulator of feeding behaviour<sup>1–4</sup>. NPY has also been implicated in the modulation of mood<sup>5</sup>, cerebrocortical excitability<sup>6</sup>, hypothalamic–pituitary signalling<sup>7</sup>, cardiovascular physiology<sup>1,8</sup> and sympathetic function<sup>9,10</sup>. However, the biological significance of NPY has been difficult to establish owing to a lack of pharmacological antagonists. We report here that mice deficient for NPY have normal food intake and body weight, and become hyperphagic following food deprivation. Mutant mice decrease their food intake and lose weight, initially to a greater extent than controls, when treated with recombinant leptin. Occasional, mild seizures occur in NPY-deficient mice and mutants are more susceptible to seizures induced by a GABA (γ-aminobutyric acid) antagonist. These results indicate that NPY is not essential for certain feeding responses or leptin actions but is an important modulator of excitability in the central nervous system.

The NPY gene was disrupted in embryonic stem cells by homologous recombination using a targeting vector in which exon 2, encoding the signal peptide and 35 amino acids of NPY, was replaced by the *lacZ* reporter gene and a neomycin-resistance cassette (Fig. 1*a*). Mice heterozygous for NPY were generated from three correctly targeted clones and bred to create mice of all possible genotypes (Fig. 1*b*). Assaying for β-galactosidase activity with X-gal revealed prominent nuclear staining in neurons of the cerebral cortex, hippocampus, arcuate nucleus of the hypothalamus, and several brainstem nuclei (Fig. 1*d*). X-gal staining was also apparent in paravertebral sympathetic ganglia, dorsal spinal cord, and enteric neurons (not shown). The distribution of X-gal staining corresponded closely to the location of NPY-expressing cells as previously determined by immunostaining and *in situ* hybridization<sup>1</sup>.

Mice homozygous for the disrupted allele do not contain detectable NPY messenger RNA in brain or NPY immunoreactivity in brain or adrenal gland (Fig. 1*c, e*). Mutant mice are born at approximately the expected frequency, with over 80 NPY-deficient mice produced so far, and are viable through adulthood. Gross and histological examination of brain and other organs revealed no anomalies. Growth of mutant mice occurs at a normal rate and both male and female mutants are fertile, demonstrating that the neuroendocrine systems regulating growth and reproduction as well as the sympathetic innervation to the vas deferens are functional in the absence of NPY.

A subset of young adult NPY-deficient mice had mild seizures, characterized by jerking of the head and body, tail erection and vocalization. Typically, these episodes lasted ~60 s and were witnessed when mice were placed on the cage top. On several occasions, mice fell over and exhibited forelimb clonus, but recovered apparently unharmed. Seizures occurred in 8 of 28 mutants and none of 40 littermate controls over a 10-week period in which each mouse was placed on the cage top for at least 5 min each week. Seizures usually occurred in 6–8-week-old mice and then remitted as they aged, suggesting that NPY exerts an inhibitory influence on neural excitability that is especially critical at that time of development. No other behavioural modifications were obvious in NPY-deficient mice.

Although seizures were not observed in mice older than 9

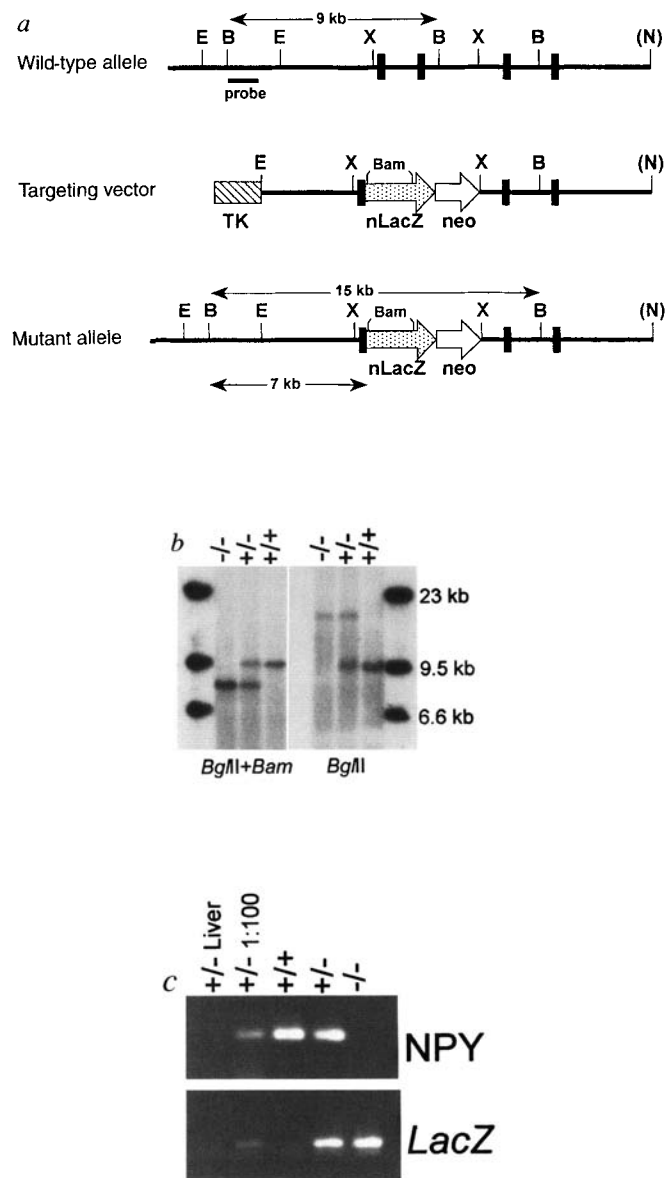


FIG. 1 Targeted disruption of the NPY gene in mice. **a**, Representation of the mouse NPY gene, the targeting vector NPYK04, and the expected targeted allele. Thick vertical bars represent exons. B, *Bgl* II; E, *Eco* RI; (N), *Not* I in polylinker; X, *Xho* I. **b**, Southern blot analysis of tail DNA from littermates. **c**, RT-PCR detection of NPY and *lacZ* mRNA in brain. **d**, X-gal staining of heterozygote brain. Left panel, coronal view showing dark, punctate staining in cortex (cx), hippocampus (hp), and hypothalamus (hy); arrow, arcuate nucleus. Right, higher magnification of hypothalamus under dark-field illumination; bright areas represent positive staining in the arcuate nucleus; 3V, third ventricle. **e**, Immunostaining with antiserum against NPY. Top, adrenal; bottom, hypothalamus. md, Adrenal medulla; cx, adrenal cortex.

**METHODS.** The mouse NPY gene was isolated from a 129 Sv genomic library using a rat NPY cDNA as a probe. Details of the construction of NPYK04 are available upon request. A *lacZ* reporter gene with a nuclear localization signal<sup>28</sup> was inserted immediately upstream of the translation initiation codon. Electroporation and selection of AB1 embryonic stem cells was performed as described<sup>29</sup>. Colonies were screened for targeting by Southern blot hybridization after digestion of DNA with *Bgl* II using a 1.7-kb *Bgl*II-HindIII fragment located 5' of the targeting vector as a probe: 15/144 clones were positive. Three lines of mice carrying the disrupted NPY allele were generated as described<sup>29</sup> from 3 different clones. F<sub>2</sub> hybrids from the 3 lines were used in all experiments. RT-PCR was performed as described<sup>29</sup>. Details of PCR and primer sequences for NPY and *lacZ* are available on request. X-gal staining was done as described<sup>28</sup>. Immunohistochemistry followed a standard streptavidin-peroxidase procedure using a 1:1,000 dilution of rabbit antiserum to human and rat NPY (Peninsula Labs).

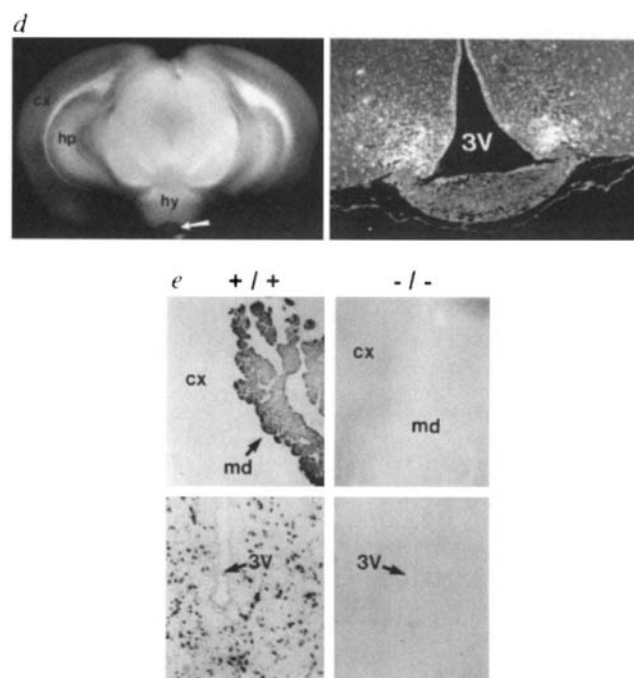


FIG. 2 NPY-deficient mice are more susceptible to seizures induced by pentylentetrazole (PTZ). **a**, Response of mice treated with 60 mg kg<sup>-1</sup> PTZ was rated on a scale from 0 to 3 depending on the most advanced stage of the seizure. A score of zero was given to mice that showed no signs of motor seizure activity, 1 for isolated twitches, 2 for tonic-clonic convulsions, and 3 for tonic extension or death. Mutant mice ( $n = 15$ ) tended to progress to more severe stages than littermate controls ( $n = 15$ ) ( $P < 0.02$ , Mann-Whitney rank sum test). **b**, Latency to seizure was shorter for mutant mice; \* $P < 0.01$ , unpaired *t*-test. Mice that did not develop seizures were assigned a latency of 488 s, equal to the longest latency observed. **METHODS.** PTZ (Sigma) was dissolved in phosphate-buffered saline and injected i.p. at a dose of 60 mg kg<sup>-1</sup> in ~0.1 ml. Each mouse was placed in a transparent cage and observed for 30 min after injection. All mice were males between 10 and 14 weeks of age.

weeks, mature NPY-deficient mice were hyperexcitable, as revealed by challenging them with the convulsant agent pentylentetrazole<sup>11</sup> (PTZ), a GABA antagonist. Following a single injection of PTZ at a dose of 60 mg kg<sup>-1</sup>, 80% of mutant mice but only 33% of wild-type or heterozygous littermates developed motor convulsions ( $P < 0.05$ ,  $\chi^2$  analysis). The response of each animal was also rated on a scale from 0 to 3, depending on the

severity of seizure activity. The distribution of scores for mutant mice was shifted towards increased severity compared to controls (Fig. 2a). Furthermore, the average latency to seizure of mutant mice was significantly shorter than the latency of littermate controls (Fig. 2b). A second group of mice treated with  $50 \text{ mg kg}^{-1}$  PTZ revealed similar differences between mutants and controls (data not shown).

Our observations that NPY-deficient mice are vulnerable to spontaneous and pharmacologically induced seizures are consistent with previous studies showing that NPY selectively suppresses excitatory transmission<sup>12-14</sup> and blocks epileptiform activity when applied to isolated brain slices and neuronal cultures<sup>15</sup>. The suppressive action of NPY on excitatory transmission in these preparations is thought to be due to inhibition of presynaptic glutamate release mediated by Y2 receptors on the terminals of excitatory neurons<sup>15-17</sup>. The absence of this inhibitory signal could account for the hyperexcitability of NPY-deficient mice.

NPY is generally accepted as a physiological effector of feeding

TABLE 1 White adipose tissue depot weights in NPY-deficient mice under various conditions

| Treatment      | Genotype | n  | Body weight (g) | Combined fat pads (g) | Per cent fat pad* |
|----------------|----------|----|-----------------|-----------------------|-------------------|
| None           | +/-, +/- | 14 | 29.85 ± 0.86    | 0.809 ± 0.088         | 2.69 ± 0.25       |
|                | -/-      | 14 | 29.10 ± 0.79    | 0.707 ± 0.063         | 2.44 ± 0.22       |
| Fast (2 d)     | +/-, +/- | 8  | 24.20 ± 0.88    | 0.345 ± 0.060         | 1.39 ± 0.20       |
|                | -/-      | 7  | 24.67 ± 0.78    | 0.276 ± 0.093         | 1.11 ± 0.35       |
| h-Leptin (5 d) | +/-      | 8  | 24.97 ± 0.73    | 0.364 ± 0.091         | 1.48 ± 0.37       |
|                | -/-      | 8  | 25.30 ± 0.85    | 0.280 ± 0.070         | 1.06 ± 0.23       |

Mice were treated as described in Fig. 3. Inguinal, epididymal, and retroperitoneal fat pads were weighed individually. The summed weights of the 3 pairs of fat pads is shown.

\* Per cent fat pad = (combined fat pad weight/body weight) × 100. Values are the mean ± s.e.m. Food deprivation for 2 d and human leptin treatment for 5 d significantly reduced body weight, fat-pad weight and per cent fat in mutant mice and control mice ( $P < 0.01$ ). No significant differences between mutant mice and control mice were observed with any treatment. All mice were males between 11 and 15 weeks of age.

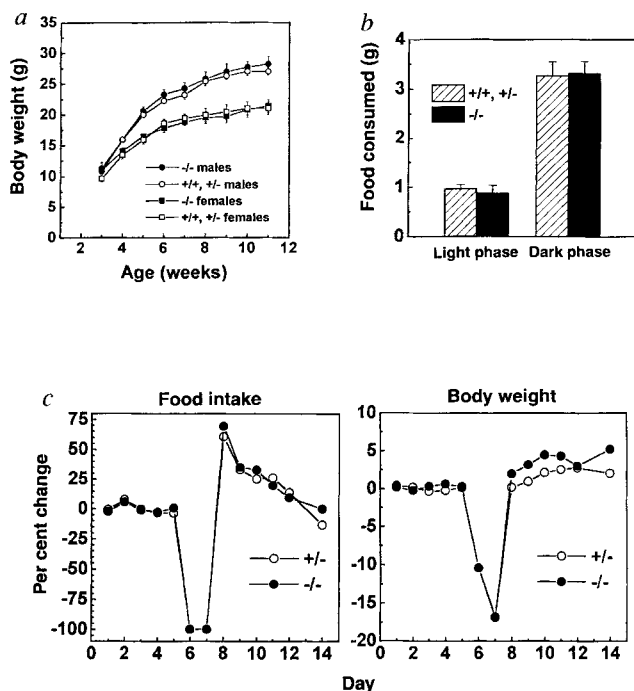
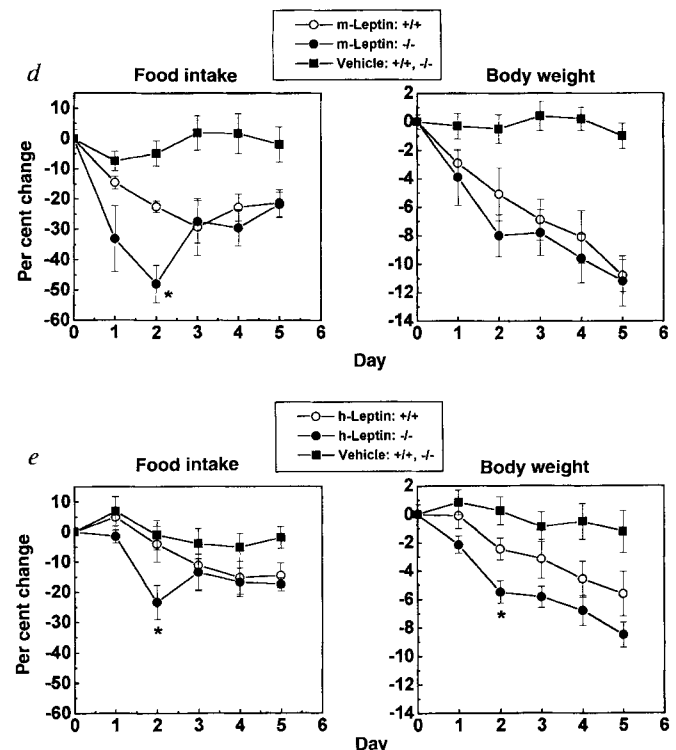


FIG. 3 Food intake and body weight of NPY-deficient mice (-/-) under various conditions. *a*, Growth curves ( $n = 6-8$ ). *b*, Food consumed *ad libitum* by female mice during the light and dark phases. Values are the mean ± s.e.m. ( $n = 8$ ). *c*, Response of NPY-deficient mice ( $n = 11$ ) and heterozygotes ( $n = 11$ ) to 48 h of food deprivation. Animals fed *ad libitum* except on days 6 and 7 when no food was available. Values (means) are plotted as per cent change from 5-day baseline values. *d*, Response of NPY-deficient female mice to murine leptin. Values (mean ± s.e.m.) are plotted as per cent change from a 7-day baseline. Mutants ( $n = 6$ ) and controls ( $n = 6$ ) decreased food intake and lost weight over the 5-d treatment period, whereas vehicle-injected mice (4 mutants and 5 controls) did not deviate significantly from baseline. Leptin suppressed feeding on day 2 to a greater extent in mutant mice;  $*P < 0.05$ . *e*, Response of NPY-deficient male mice to human leptin. Human leptin suppressed food consumption

behaviour because it potently induces feeding when administered into the brain<sup>18,19</sup>, and its synthesis and release in the hypothalamus are coupled to metabolic status<sup>20-22</sup>. Remarkably, food intake, body weight and adiposity were normal in NPY-deficient mice (Fig. 3a, b; Table 1). Serum glucose, insulin and corticosterone levels were also normal (data not shown). Because NPY has been proposed to mediate fasting-induced hyperphagia<sup>21-24</sup>, we examined the response of mice to 48 h of food deprivation. The resultant stimulation of food intake in mutant mice was similar in



and induced weight loss in mutants ( $n = 8$ ) and controls ( $n = 8$ ). Mutant mice displayed a greater response than controls on day 2;  $*P < 0.05$ .

**METHODS.** Mice were maintained under 12 h light/12 h dark conditions and fed standard mouse chow (Teklab). Mature murine and human leptin were made in yeast as secreted and cytosolic forms, respectively, and purified by 2-step chromatography. Purity was >95% by analytical HPLC. Leptin was stored in 50 mM  $\text{Na}_2\text{BO}_4$ , pH 7.5, and diluted in saline before injection. Vehicle consisted of identically processed preparations from yeast lacking the coding region for leptin. Leptin ( $45 \mu\text{g}$ ) or vehicle were injected intraperitoneally ~1 h before the dark phase and again ~6 h later. Human leptin may have been less active than murine leptin because it was denatured/refolded during purification and underwent several freezings. Moreover, human leptin was administered to ~30-g males, whereas murine leptin was given to ~22-g females.



extent and duration to that in controls, and both mutants and controls rapidly recovered lost body mass after this challenge (Fig. 3c). These observations demonstrate that NPY is not required for appropriate feeding under basal conditions or after food deprivation. Careful examination of other peptides, transmitters, hormones and pathways within the hypothalamus will be required to determine whether normal feeding behaviour in NPY-deficient mice is due to redundancy in systems that normally control food intake or to compensation by other systems in a manner unique to NPY-deficient animals.

Leptin, a hormone secreted by adipocytes that is essential for normal body weight regulation<sup>25</sup>, has been proposed to suppress feeding and decrease adiposity through its ability to inhibit hypothalamic NPY synthesis and release<sup>26,27</sup>. To assess the consequences of NPY deficiency on leptin signalling, we treated mice with recombinant murine or human leptin for 5 days. Leptin, but not vehicle, significantly reduced food intake, body weight and adipose tissue mass in both mutant and control mice (Fig. 3d, e; Table 1). NPY-deficient mice displayed a greater initial reduction in food intake and tended to lose more weight over the first 2 d but resembled controls for the remainder of the treatment period. These results demonstrate that leptin can suppress feeding and promote weight loss via signalling pathways that are either independent of, or additional to, those using NPY. Moreover, the increased sensitivity of NPY-deficient mice to acute elevations in leptin implies that NPY opposes leptin's inhibitory effect on food intake. Inhibition of NPY synthesis and release by leptin in normal mice could account for the similar response of mutants and controls after several days of treatment. Alternatively, weight loss in mutant mice might have activated other compensatory feeding systems to counter the initial pronounced suppression of food intake induced by leptin. NPY-deficient mice will be useful in determining the role of NPY in obesity syndromes. □

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## Rat spermatogenesis in mouse testis

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**RECENTLY**, transplantation of mouse donor spermatogonial stem cells from a fertile testis to an infertile recipient mouse testis was described<sup>1,2</sup>. The donor cells established spermatogenesis in the seminiferous tubules of the host, and normal spermatozoa were produced. In the most successful transplants, the recipient mice were fertile and sired up to 80 per cent of progeny from donor cells<sup>2</sup>. Here we examine the feasibility of transplanting spermatogonial stem cells from other species to the mouse seminiferous tubule to generate spermatogenesis. Marked testis cells from transgenic rats were transplanted to the testes of immunodeficient mice, and in all of 10 recipient mice (in 19 of 20 testes), rat spermatogenesis occurred. Epididymides of eight mice were examined, and the three from mice with the longest transplants ( $\geq 110$  days) contained rat spermatozoa with normal morphology. The generation of rat spermatogenesis in mouse testes suggests that spermatogonial stem cells of many species could be transplanted, and opens the possibility of xenogeneic spermatogenesis for other species.

Spermatogenesis is a complex process in which spermatogonial stem cells adjacent to the seminiferous tubule basal membrane of the testis undergo division, to renew the population of stem cells and to generate progeny cells that develop into spermatozoa simultaneously<sup>3–6</sup>. The process is thought to be regulated by the somatic Sertoli cells that line the seminiferous tubules and that support and nurture germ cells during spermatogenesis<sup>7,8</sup>. Unlike mammalian female germ cells, which cease dividing before birth, male spermatogonial stem cells continue to divide throughout adult life<sup>6,9</sup>.

To extend the spermatogonial transplantation technique to other species and assess the feasibility of using the mouse as a repository of xenogeneic spermatogenesis, we transferred donor cells from the testes of fertile transgenic Sprague–Dawley rats into the testes of immunodeficient mice sterilized by busulphan treatment. We used both nude and severe combined immunodeficient (SCID) mice as recipients, as transplantation of donor cells into immunocompetent mice (C57BL/6  $\times$  SJL F<sub>1</sub>) did not result in rat spermatogenesis. Donor rats carried the *MT-lacZ* transgene<sup>10</sup>, which was expressed in Sertoli cells and germ cells allowing them to be identified by their blue staining after incubation with X-gal (Fig. 1a). Recipient mice were killed between 42 and 127 days after cell transfer, and their testes examined for evidence of rat spermatogenesis. In rat, the length of time from initiation of stem cell division to formation of mature spermatozoa is 52 days, compared with 35 days in the mouse<sup>3,6</sup>. Thus, in these experiments, the time available for xenogeneic sperm development was 80 to 240 per cent of the time necessary for rat spermatogenesis. To identify spermatogenesis arising from rat stem cells, recipient testes were incubated with X-gal, which results in blue staining of transgenic rat but not control rat or recipient mouse germ cells (Fig. 1a, b). The seminiferous tubules of busulphan-treated recipient mice do not generally contain male germ cells (Fig. 1c). Furthermore, any endogenous mouse germ cells would not stain blue, because they do not carry the

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TABLE 1 Rat spermatogonial stem cell transfer into mouse recipient testes

| Experiment | Recipient mouse | Genotype | Per cent testis surface area covered*<br>right, left | Time to analysis, days† | Number of testis tubules with donor cells‡<br>right, left | Rat sperm in epididymides§ |
|------------|-----------------|----------|------------------------------------------------------|-------------------------|-----------------------------------------------------------|----------------------------|
| 1          | 761             | SCID     | 50, 25                                               | 46                      | 10, 10                                                    | ND                         |
|            | 763             |          | 50, 50                                               | 97                      | 5, 4                                                      | 0/852                      |
| 2          | 766             | Nude     | 80, 80                                               | 91                      | 8, 3                                                      | 0/399                      |
|            | 767             |          | 80, 90                                               | 110                     | ≥ 12, ≥ 12                                                | 7/270                      |
| 3          | 769             | SCID     | 60, 25                                               | 42                      | 4, 0                                                      | ND                         |
|            | 770             |          | 50, 30                                               | 104                     | ≥ 12, ≥ 12                                                | 1/1832                     |
| 4          | 775             | Nude     | 90, 50                                               | 127                     | ≥ 12, ≥ 12                                                | 24/1273,<br>4/2041         |
|            | 776             |          | 80, 50                                               | 77                      | 3, 4                                                      | 0/1284                     |
|            | 777             |          | 90, 90                                               | 121                     | 8, ≥ 12                                                   | 3/2370                     |
|            | 778             |          | 50, 50                                               | 96                      | 8, 6                                                      | 0/189                      |
|            |                 |          |                                                      |                         |                                                           |                            |

SCID mice, which lack B-cells and T-cells. Nude, Swiss nude mice, which lack T-cells. Recipient mice received 32 mg per kg busulphan, which destroys endogenous spermatogenesis but allows partial regeneration with time. This system provides carrier mouse spermatozoa and results in a mixed population of spermatozoa in the epididymis<sup>2</sup>. Transgenic rat donor testis cells were collected and microinjected into the seminiferous tubules of recipient mice as previously described<sup>2</sup>: Experiment 1,  $40 \times 10^6$  cells per ml from 6 hemizygous transgenic 17-day-old rat testes; Experiment 2,  $79 \times 10^6$  cells per ml from 6 hemizygous transgenic and 6 control 12-day-old testes; Experiment 3,  $65 \times 10^6$  cells per ml from 4 hemizygous transgenic and 8 control 12-day-old testes; Experiment 4,  $59 \times 10^6$  cells per ml from 4 hemizygous transgenic and 4 control 13-day-old testes. Roughly 0.5 ml of cell suspension was used to inject each mouse.

\* Percentage of the surface seminiferous tubules in recipient testes filled by the injected rat cell suspension.

† Number of days from injection of donor cells to analysis of the recipient testis for presence of spermatogenesis; the time available for spermatogenesis.

‡ Number of mouse seminiferous tubules with evidence of rat spermatogenesis as indicated by blue staining (Fig. 1). Tubules in testes with more than 12 stained could not be counted accurately because of convolution of the tubules<sup>2</sup>.

§ ND, not determined. Numerator, number of rat spermatozoa; denominator, number of mouse spermatozoa. In all cases except 775, the spermatozoa from both epididymides were pooled. In 775 the first number is for right, the second for left. Spermatozoa were identified by characteristic morphology (see Fig. 3).

|| Rat spermatozoa had normal tail and small round head. Epididymides were fixed in neutral buffered formalin before dissection, which makes release of spermatozoa more difficult.

transgene<sup>1,2</sup>. Mouse seminiferous tubules in which rat spermatogenesis occurred were stained blue from the earliest time point examined, indicating that rat testis cells survived isolation and transplantation and could subsequently populate mouse seminiferous tubules (Fig. 1d, Table 1).

To determine the efficiency of colonization of mouse seminiferous tubules by rat testis cells, microscopic sections of recipient testes were examined. In transgenic rat donor testes, Sertoli cells and germ cells expressed  $\beta$ -galactosidase and stained blue (Figs 1a, 2a). There was no staining in non-transgenic rat testes (Figs 1b, 2b) or unrepopulated immunodeficient busulphan-treated mouse

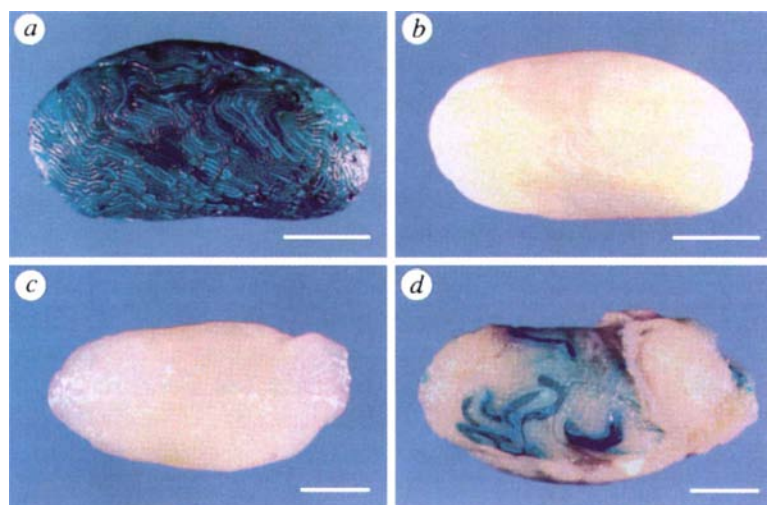
testes (Figs 1c, 2c). However, areas in recipient testes colonized by transgenic donor testis cells were stained blue. As in donor rats, germ cell stages were stained (Fig. 2d, e and f). Mature forms of rat spermatozoa could also be identified, displaying the characteristic long, thin rat spermatozoa head (compare Fig. 2d, e and f with Fig. 2a and b). Although the donor cell population contained both Sertoli cells and germ cells, blue-stained rat Sertoli cells were not seen in the recipient tubules (Fig. 2d, f), suggesting that they did not colonize the recipient tubule and that rat spermatogenesis was being supported by mouse Sertoli cells.

Spermatozoa were recovered from the epididymides of eight of

FIG. 1 Generation of rat spermatogenesis in mouse testes.

a, Testis from 8-week-old transgenic rat (line 14-2) incubated with 5-bromo-4-chloro-3-indole- $\beta$ -D-galactosidase (X-gal). The transgene was composed of the mouse metallothionein-I promoter and flanking regions (MT) fused to the *Escherichia coli lacZ* (*lacZ*) structural gene, which results in expression of  $\beta$ -galactosidase ( $\beta$ -gal) in the liver and testes of rats, causing blue staining of these tissues after incubation with X-gal (refs 1,2,10 and unpublished results). In rats, Sertoli cells are uniformly stained (light areas) and all germ cells will stain blue depending on substrate penetration (dark areas). b, Testis from 8-week-old non-transgenic rat which does not stain blue after incubation with X-gal. c, Testis from 16-week-old immunodeficient nude (nu/nu) mouse treated with busulphan (32 mg per kg) to destroy endogenous mouse spermatogenesis<sup>1,2,16</sup>. d, Testis from busulphan-treated nude mouse that was injected with transgenic rat testis cells (Left testis of 777, Table 1). Enlarged area is site of injection from which some tubules were extruded. Blue tubules represent areas of rat spermatogenesis in the mouse testis. Scale bars: a, b, 5 mm; c, d, 1 mm.

METHODS. Transgenic rats were generated as previously described<sup>17</sup>. Technique for isolating testis cells<sup>18,19</sup>, microinjection into recipient mouse seminiferous tubules and analysis of testes have also been described<sup>1,2</sup>. Roughly 0.5 ml of cell suspension was used to inject the



testes of each recipient. Recipient Swiss nude mice were injected with bone marrow cells ( $\sim 3 \times 10^6$  per mouse) into the tail vein, 3 days after busulphan treatment, to reduce mortality.



the ten recipient mice and examined using phase contrast and fluorescence microscopy (Fig. 3a–f). In the best recipient mouse (767, Table 1), roughly one in 39 epididymal spermatozoa arose from rat donor cells, and in the right epididymis from recipient 775, one in 53 spermatozoa were from rat. The three recipients killed at the longest time post-transplantation ( $\geq 110$  days) contained normal rat spermatozoa in the epididymides (Table 1). In a fourth animal killed after 104 days, a rat spermatozoon with a normal tail and an abnormal head was found. Thus, appearance of rat spermatozoa in the epididymides is favoured by a long period of colonization ( $\geq 104$  days), extensive colonization of recipient seminiferous tubules ( $\geq 12$  tubules), and use of nude rather than SCID recipients.

Although the general process of spermatogenesis is highly conserved, many characteristics, including timing of the differentiation process and spermatozoa morphology, are unique and species-specific<sup>3–6</sup>. Thus, the capacity of the mouse seminiferous tubule to support production of spermatozoa that normally

require 50 per cent longer to develop and have a different morphology is remarkable. Whereas Sertoli cells were present with transplanted donor germ cells and may be necessary to support rat spermatogenesis, the absence of blue-staining rat Sertoli cells in association with rat spermatogenesis in recipient mice suggests considerable flexibility in the supporting role of the Sertoli cell and in its interaction with the differentiating stages of male germ cells. As the mouse and rat are believed to have diverged 11 million years ago<sup>11</sup>, this reflects a striking conservation of a very specific and highly organized process.

A practical application of our results may be to establish the mouse as an *in vivo* host for spermatogenesis of other species. Immunodeficient mice could prove useful for developing spermatozoa from subfertile or valuable males, in species where immunologically tolerant recipients are not available. It may not be essential to produce many spermatozoa from the transplanted donor cells or for the final stages of maturation to be completed, as it has been demonstrated that round spermatids as well as mature

FIG. 2 Microscopic appearance of rat spermatogenesis in mouse seminiferous tubules. *a*, Seminiferous tubule of transgenic (*MT-lacZ*) rat. Sertoli cells along basement membrane are uniformly stained. All germ cell stages stain depending on substrate penetration. Centre tubule shows staining of pachytene spermatocytes. Stain, X-gal and neutral fast red (NFR). *b*, Seminiferous tubule of control rat. No cells are stained blue by X-gal. The long, thin heads of mature rat spermatozoa are evident. Stain, X-gal and NFR. *c*, Seminiferous tubule of nude mouse treated with busulphan. No germ-cell stages are present; only Sertoli cells can be seen on the basement membrane of the tubule. Stain, NFR. *d*, Seminiferous tubule of busulphan-treated recipient nude mouse showing rat spermatogenesis from transplanted cells (left testis from mouse 777). Rat pachytene spermatocytes and round spermatids are heavily stained blue. Sertoli cells (arrowheads) do not stain blue. Stain, X-gal and NFR. *e*, Seminiferous tubule from left testis of mouse 777. Rat preleptotene and pachytene spermatocytes are stained blue. Stain, X-gal plus periodic acid Schiff and haematoxylin, which emphasizes acrosome and head shape of rat spermatozoa. *f*, Seminiferous tubule from left testis of mouse 775. Rat preleptotene and pachytene spermatocytes are stained blue. Sertoli cells (arrowheads) do not stain blue. Stain, X-gal plus haematoxylin and eosin to emphasize normal morphology of spermatogenesis arising from transplanted rat stem cells. Scale bar, 50  $\mu$ m.

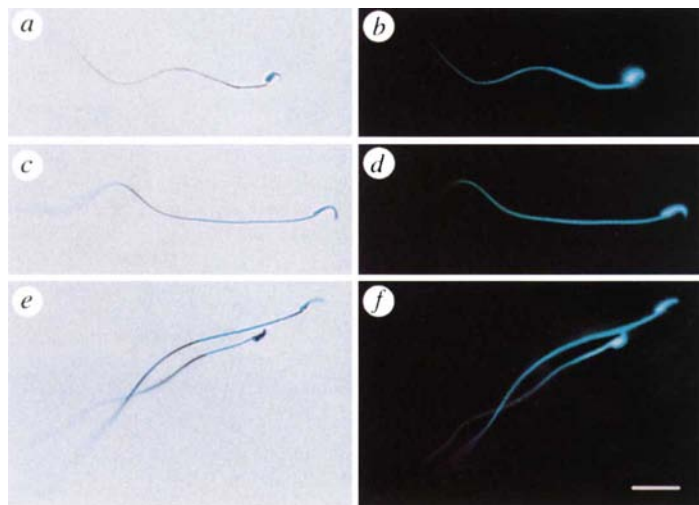
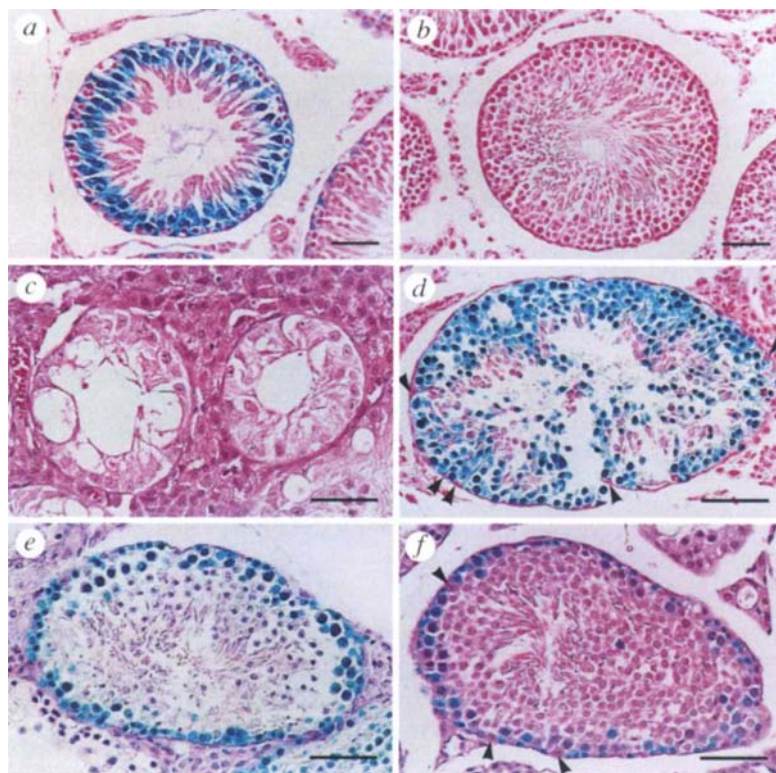


FIG. 3 Epididymal spermatozoa. *a*, *c* and *e* are phase contrast and *b*, *d* and *f* are fluorescence photomicrographs. *a*, *b* Normal mouse spermatozoa, with short, thick, sickle-shaped head. *c*, *d* Normal rat spermatozoa, with long, thin head, and tail that is longer and thicker than mouse. *e*, *f* Spermatozoa from epididymides of recipient mouse 767, with distinct rat and mouse morphologies. Ratio was 1 rat to 39 mouse spermatozoa in this animal.

**METHODS.** To examine and count spermatozoa, epididymides were placed in phosphate-buffered saline and cut in several places<sup>20</sup>. Mouse and rat spermatozoa were distinguished by their characteristic head morphology and tail size. For fluorescence photography, the spermatozoa solution was treated with 20  $\mu$ g per ml Hoechst 33258 dye<sup>20</sup>. Scale bar, 25  $\mu$ m.



spermatozoa can fertilize eggs after intracytoplasmic injection in mice and humans<sup>12-14</sup>. Finally, although culture systems are not currently available for spermatogonial stem cells, if this were achieved, modification of the genes in these cells should be feasible. In combination with xenogeneic spermatogonial transplantation, this would facilitate the extension of the full range of transgenic technology<sup>15</sup> to many, if not all, mammalian species. Because of its ability to replicate throughout life, the male germ line stem cell may prove useful in biology, medicine and agriculture.

**Note added in proof:** 'Intraluminal spermatogenesis' after transfer of male germ cells from Sprague-Dawley rats to the seminiferous tubules of busulphan-treated Long-Evans rats has recently been described<sup>21</sup>. Spermatogonial stem cells can now be cryopreserved, making the germ line of individual males immortal<sup>22</sup>. □

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## A gene complex acting downstream of *dpp* in *Drosophila* wing morphogenesis

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**LOCALIZED expression of the transforming growth factor- $\beta$  (TGF- $\beta$ ) homologue *decapentaplegic* (*dpp*) is crucial for *Drosophila* wing development<sup>1-4</sup>. Here we show that *spalt* and *spalt-related* (*sal* and *salr*), two closely related genes that encode transcription factors, are expressed in response to *dpp* in a central territory of the wing imaginal disc, where they are required for the patterning of the wing. They are among the first identified elements that act downstream of *dpp* in wing development. The phenotypic consequences of misexpression of *sal* and *salr* suggest that an important outcome of *dpp* activity is the subdivision of the wing disc into territories smaller than lineage compartments, through the regulation of transcription-factor-encoding genes such as *sal* and *salr*.**

The *Drosophila* genes *sal* and *salr* are located approximately 65 kilobases apart and encode proteins with related DNA-binding zinc-fingers<sup>5,6</sup>. Expression of *sal* and *salr* is first detectable in the wing disc early in the third larval instar, initially dorsally and then in a broad, dorsoventrally elongated zone that straddles the anteroposterior (A/P) compartment boundary<sup>7</sup> and is symmetrical with respect to the dorsoventral axis (Fig. 1a-f). The domain of expression of *sal* and *salr* is coincident (Fig. 1g-i) and is centred on the *dpp* stripe (Fig. 1e). The expression of the two genes in the imaginal disc corresponds to the region of the adult wing between

vein LII and the intervein LIV-LV (sectors B, C and part of D; Figs 1d and 2a, j).

To determine the functional significance of *sal* and *salr*, we examined mosaic wings bearing mitotic clones of a small deficiency (*Df5*) that removes both genes but not other transcripts expressed in imaginal discs (data not shown). Depending on clone location, we observe considerable reorganization of the vein pattern, including disappearance, displacement, fusion and ectopic formation of specific veins, as well as reductions in wing size (Fig. 2). Even large clones of *Df5* (generated in a *Minute* mutant background<sup>8</sup>) only have phenotypic consequences if they overlap the domain where *sal* and *salr* are expressed. However, such clones also affect veins that lie outside the expression domain of *sal* and *salr*, namely LII and LV. Thus clones including sector D cause anterior displacement or ectopic spurs of LV, or its fusion with LIV (Fig. 2c, f, i); ventral clones in sector B lead to loss of the LII vein, especially proximally (Fig. 2d, e). These effects suggest that the boundary between cells with and cells without *sal* and *salr* expression may be used during normal development to establish the position of veins LII and LV. Within the expression domain of *sal* and *salr* itself, LIII is displaced anteriorly or posteriorly when the clones are in sector B or C, respectively, and LIV is displaced posteriorly when the clones are in sector D (Fig. 2c, j-m). The modified positions of these veins are associated with reductions in the size of the affected sectors, suggesting that within their domain of expression *sal* and *salr* are needed for both vein patterning and cell growth. The requirement of the genes in cell growth is most obvious when the proliferation abilities of wild-type and *Df5* mutant cells are compared in a 'twin clone' analysis. Thus, in sectors B, C and D (but not in sectors A and E), the *Df5* clones are reduced in size compared with their wild-type twins, or are completely absent (Fig. 2).

The genes *sal* and *salr* seem to have at least partly redundant functions because clones of a null allele of *sal*, *sal<sup>445</sup>*, result in weaker effects than those observed in comparable *Df5* clones, with respect to both size reduction and vein displacement (Fig. 2g). However, *sal<sup>445</sup>* clones in sector B cause an ectopic LII vein and displace it towards LIII (Fig. 2h), again suggesting that a boundary between cells that do and those that do not express *sal* determines LII localization. Large clones of *In(2L)FCK73*, a strong hypomorphic allele of *salr* that reduces its transcription (F.C.K. et al., manuscript in preparation) do not produce any alteration in the

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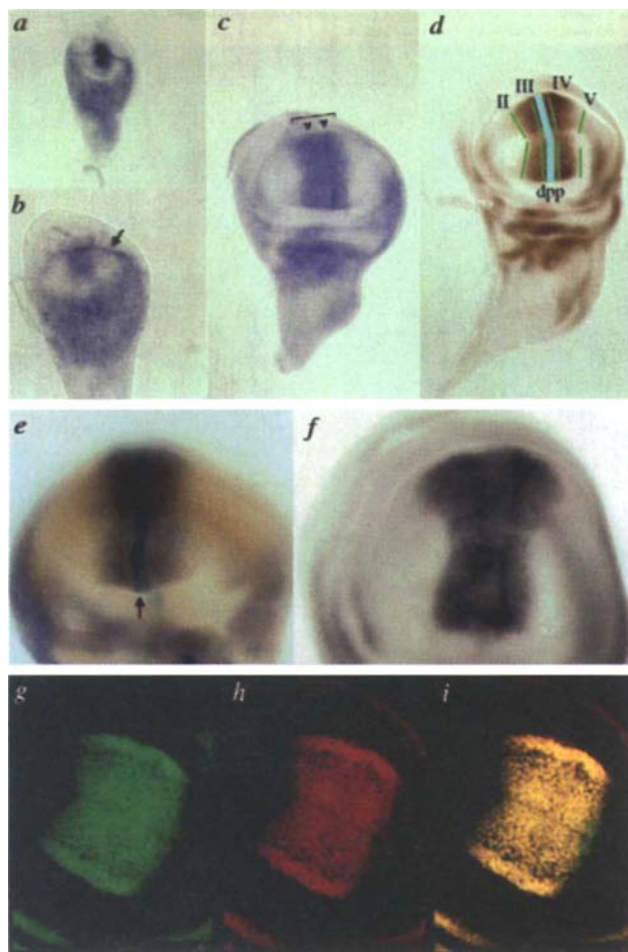


FIG. 1 Expression pattern of *sal* and *salr* in the wing imaginal disc. *a, b*, *In situ* hybridization with *wingless* and *salr* probes reveals that, in early third-instar discs, expression of *salr* in the presumptive wing blade is limited to a narrow band 4–6 cells wide, perpendicular to the dorsoventral boundary (here identified by *wingless* expression; arrow in *b*). The band is first detected in the dorsal compartment (*b*) and then expands ventrally (*a*). In late third-instar discs the expression of *sal* (*c, d*) and *salr* (*e, f*) occurs in a broader domain centred on the *dpp* stripe (blue counter-stain in *e*, arrow). Both *in situ* hybridization (*c* and *e*) and antibody staining (*d* and *f*) shows that the wing-blade *sal/salr* domain (bracket in *c*) includes anterior and posterior stripes where the expression is stronger (arrowheads in *c*). In *d*, the positions of the veins (II, III, IV and V) and the *dpp* stripe (*dpp*) are depicted by green lines and blue line, respectively. The approximate locations of presumptive LII, LIII, LIV and LV veins were defined as in Fig. 2j. Confocal images of a late third-instar disc stained with antibodies to visualize *sal* protein (H, red) and *salr* protein (G, green) reveal that the expression domains of these proteins are coincident (I, yellow). Expression also can be seen outside the wing-blade region, but is not considered here. Magnification in *b, e* and *f* is the same, and double that in *a, c* and *d*.

METHODS. Whole-mount *in situ* hybridization with digoxigenin-labelled DNA probes, immunocytochemistry and X-gal staining were as described<sup>17</sup>. Gene-specific hybridization probes were a 547-bp-long *Pst*I–*Pvu*II cDNA fragment, corresponding to the region between fingers 5 and 6 of the *salr* protein (R.B. *et al.*, manuscript in preparation), and a 437-bp-long *Sty*I fragment, representing the corresponding inter-finger region in *sal* protein<sup>5</sup>. A 0.8-kb *Eco*RI fragment from the *wingless* cDNA clone wgc14 (ref. 18) and a 2-kb *Eco*RI *dpp* cDNA fragment (Fig. 4) were also used as probes. We used *dpp-lacZ* line BH3.1 (ref. 19). Immunocytochemistry was performed with rabbit anti-Sal, kindly provided by R. Schuh<sup>2</sup>, and rat anti-Salr (R.B. *et al.*, manuscript in preparation) as primary antibodies. Secondary antibodies were from Vector.

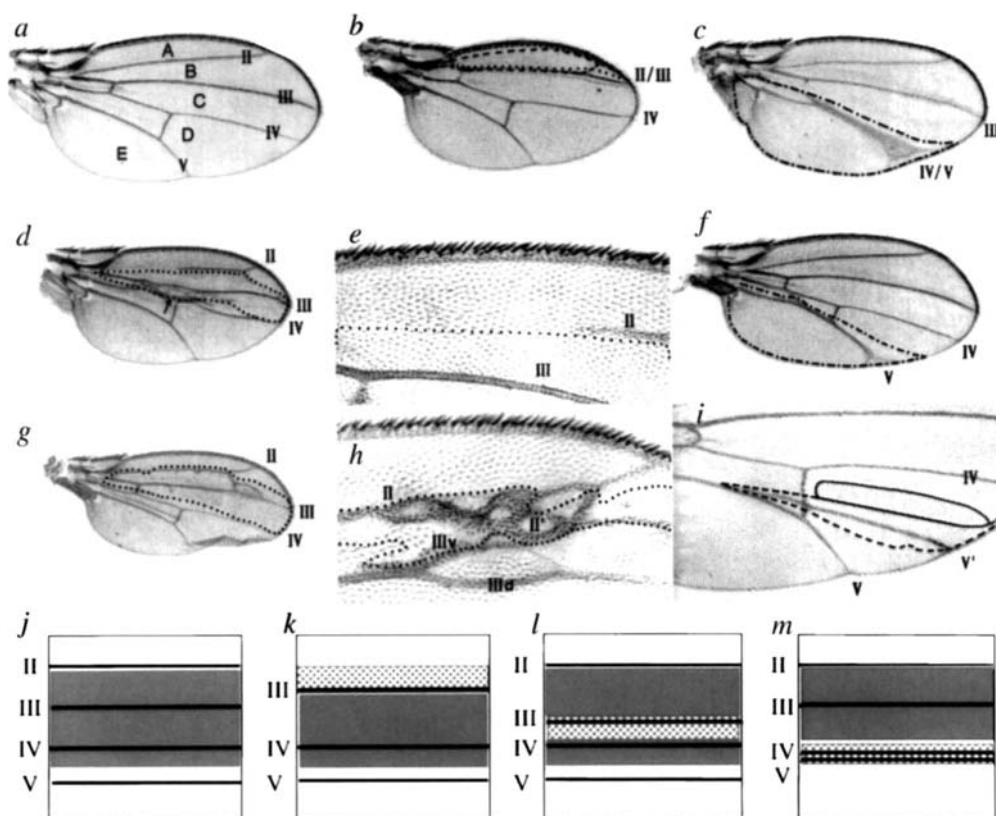


FIG. 2 Mosaic analysis of *sal* and *salr*. *a*, Wild-type wing identifying the veins LII–LV and the wing sectors A–E that they delimit. *b–f*, *Minute*<sup>+</sup> clones (dotted, ventral; dashed, dorsal) homozygous for *Df5*, a deficiency lacking both *sal* and *salr*, grow to occupy large wing territories but show defective vein patterns. Remnants of vein LII are fused with LIII in the largest dorsoventral anterior clones (*b*), and LIV and LV are fused in the largest dorsoventral clones in the posterior compartment (*c*). Note the associated reduction in wing size. Ventral clones in the anterior compartment close to LII remove the proximal part of this vein (*d, e*), and when they occupy a large fraction of the compartment the opposite wing surface is folded (*d*, arrow). Posterior compartment clones with a boundary between LIV and LV show anterior displacement of LV (*f*) and

vein pattern (data not shown). The identical expression patterns of *sal* and *salr*, their sequence similarity, and the finding that the strongest phenotypes are only observed when both genes are deleted, suggest that they form a gene complex similar to other regulatory gene complexes in *Drosophila*, notably the *engrailed/invected* and *achaete/scute* complexes. In all three cases, extreme phenotypes are only observed after removal of both genes in the complex, although one of the partners appears to be more critical than the other<sup>9,10</sup>.

Expression patterns of *sal* and *salr*, and the phenotypes of *Df5* in mosaic wings, strongly suggest that these genes have a role in anteroposterior patterning. Because *dpp* is the major element in A/P patterning<sup>1</sup>, we examined the possibility that it regulates

these genes (Fig. 3). Elimination of *dpp* expression in the wing blade (*dpp<sup>Δ5</sup>* discs) results in extremely reduced wings; in the corresponding discs the expression of *sal* and *salr* is abolished in the presumptive region of the wing blade (Fig. 3d, f). Weaker *dpp* heteroallelic combinations result in wing phenotypes very similar to those of *Df5* clones, including absence of LII, fusion of LII with LIII, and fusion of LIV with LV (Fig. 3e). In this case, decreased *dpp* activity is correlated with a reduction in *sal* and *salr* expression (Fig. 3g; compare with Fig. 1f). Conversely, ectopic *dpp*, either throughout the anterior compartment (*hh<sup>Mtr</sup>* homozygous mutant discs<sup>11</sup>) or in both compartments (GAL4 enhancer trap lines crossed with a UAS-*dpp* strain; Fig. 3b), results in corresponding ectopic expression of *sal* and *salr* (Fig. 3a, c). Taken together,

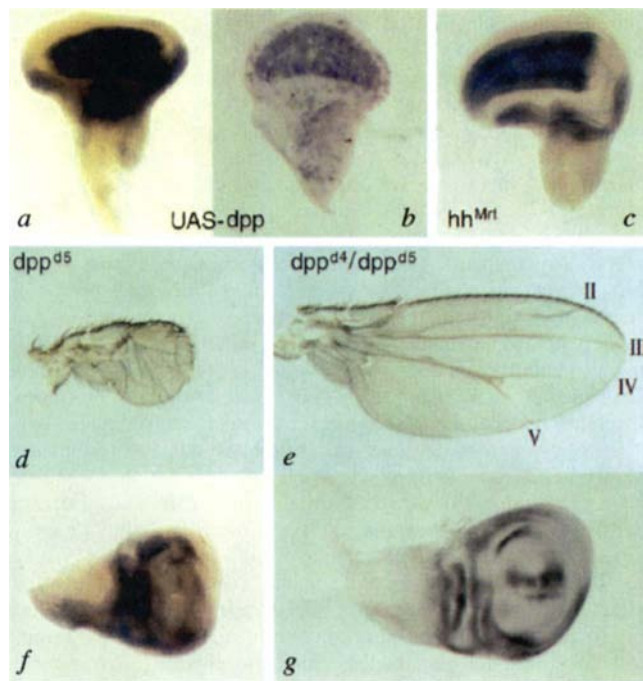


FIG. 3 The genes *sal* and *salr* are regulated by *decapentaplegic*. a, b, Correlation of *dpp* and *salr* expression patterns. Ectopic expression of *dpp* in the combination GAL4-580/UAS-*dpp* occurs throughout the wing blade, as revealed by *in situ* hybridization with a *dpp* probe (b); it results in dramatic wing overgrowth accompanied by expansion of *salr* expression to fill most of the presumptive wing blade (a). In *hedgehog<sup>Moonrat</sup>* (*hh<sup>Mtr</sup>*) homozygous discs, *salr* is expressed throughout the expanded anterior compartment (c), in a pattern very similar to that documented for *dpp* in the same discs<sup>11</sup>. The results of a and c were duplicated with *sal*-specific probes and *Salr* and *Sal* antibodies (data not shown). d–g, We analysed two different *dpp* loss-of-function alleles, *dpp<sup>Δ5</sup>* in homozygosity and *dpp<sup>Δ4</sup>/dpp<sup>Δ5</sup>* in transheterozygosity. The *dpp<sup>Δ5</sup>* wings are severely reduced in size, with only proximal structures identifiable (d); in this genotype, *dpp* expression in the wing blade is absent (data not shown), and *salr* RNA expression is severely reduced or absent in the same region (f). In *dpp<sup>Δ4</sup>/dpp<sup>Δ5</sup>* wings the LII vein is underdeveloped and LIV/LV are closer and partly fused (e); in this genotype, the domain of *sal* protein expression is reduced in width, and a clear gap appears in the posterior compartment (g; see Fig. 1f).

differentiation of ectopic LV stretches; an ectopic LV segment, V', is seen (i) within a mutant clone adjacent to its wild-type twin; clones outlined by dashed and continuous lines, respectively. (g–h): Clones of *sal<sup>Δ45</sup>*, a *sal* null allele<sup>5</sup>, cause slightly weaker phenotypes than equivalent clones of *Df5* (compare d and g). Ventral clones in sector B (h) displace the LII vein posteriorly, and cause differentiation of ectopic LII stretches, II', as well as local dissociation of the LIII dorsal and ventral vein components, IIId, IIIV; they do not eliminate proximal LII, unlike *Df5* clones (e). j, The domain of *sal/salr* expression relative to the veins of wild-type adult wing. The expression of *sal/salr* in the wing-blade region corresponds to a central territory extending from just posterior to vein LII until the interval between veins LIV and LV (grey); this was revealed both using molecular probes to detect the position of the presumptive veins in the disc and by driving the expression of cell-differentiation genes such as *scute* in the *sal/salr* domain using a *sal* GAL4 line (data not shown). k–m, Diagrams of effects on the vein pattern resulting from loss of *sal/salr* expression (light-stippling) in typical *Df5* clones at different locations. The expression of *sal/salr* is eliminated from sector B in anterior clones between vein LIII and the anterior margin (k), so LII disappears and LIII is displaced anteriorly (l). When *sal/salr* expression is eliminated from clones in sector C (l, between LIII and LIV), the distance between these two veins is reduced because of growth impairment (l). In a posterior clone between LIV and the posterior margin, *sal/salr* expression is eliminated in the posterior compartment (m), so LIV and LV move towards each other and may fuse (m).

METHODS. Mitotic recombination was induced by X-rays (dose 1,000 R; 300 R min<sup>-1</sup>, 100 Kv, 15 mA, 2 mm aluminium filter). Irradiated larvae were timed in hours after egg laying (AEL). *Df5* is shorthand for *Df(2L)32FP-5*, and *In(2L)FCK73* is a strong *salr* hypomorph (F.C.K. et al., manuscript in preparation). Clones were scored in males of genotypes [*f<sup>36a</sup>*; *Ins(f<sup>+</sup>)30B M(2)Z/In(2L)FCK73*], [*f<sup>36a</sup>*; *Ins(f<sup>-</sup>)30B M(2)Z/Df5*], [*f<sup>36a</sup>*; *Ins(f<sup>-</sup>)30B ck/Df5*] and [*f<sup>36a</sup>*; *Ins(f<sup>-</sup>)30B M(2)Z/sal<sup>Δ45</sup>*]. Mitotic recombination proximal to the *f<sup>+</sup>* insertion produces homozygous mutant cells labelled with the cell marker *forked* (f). In the twin-clone experiment [*f<sup>36a</sup>*; *Ins(f<sup>+</sup>)30B ck/Df5*] the mean numbers of cells in clones induced throughout the wing at 48–72 h AEL were: 130 *f<sup>36a</sup> Df5* versus 180 *ck Df5* (20 twin clones). In sectors B and C, *ck* clones have an *f* twin in only 30% of the cases if induced early (48–72 h AEL), and in 40% of the cases if induced late (72–96 h). In sector D, 70–80% of the *ck* clones have an *f* twin clone. In sectors A and E, 95% of *ck* clones have an *f* twin of similar or larger size. In *M<sup>+</sup>* experiments we analysed 45 *f<sup>36a</sup> Df5 M<sup>+</sup>* clones, 23 *f<sup>36a</sup> sal<sup>Δ45</sup> M<sup>+</sup>* clones, and 6 *f<sup>36a</sup> In(2L)FCK73 M<sup>+</sup>* clones. In the *f<sup>36a</sup> Df5 M<sup>+</sup>* experiment pairs of appendages of the same individual were used to make morphometric comparisons in cases of mosaicism. In large dorsoventral clones the affected compartment (anterior or posterior) is reduced by 30–45% relative to the same compartment in the contralateral wing. Estimations of trichome density and number of cells in mosaic areas were done in photographic reproductions of wings using the program IMAGE (from W. Rasband, NIH, Bethesda, MD). Wings were mounted for microscopic examination in lactic acid:ethanol (1:1).



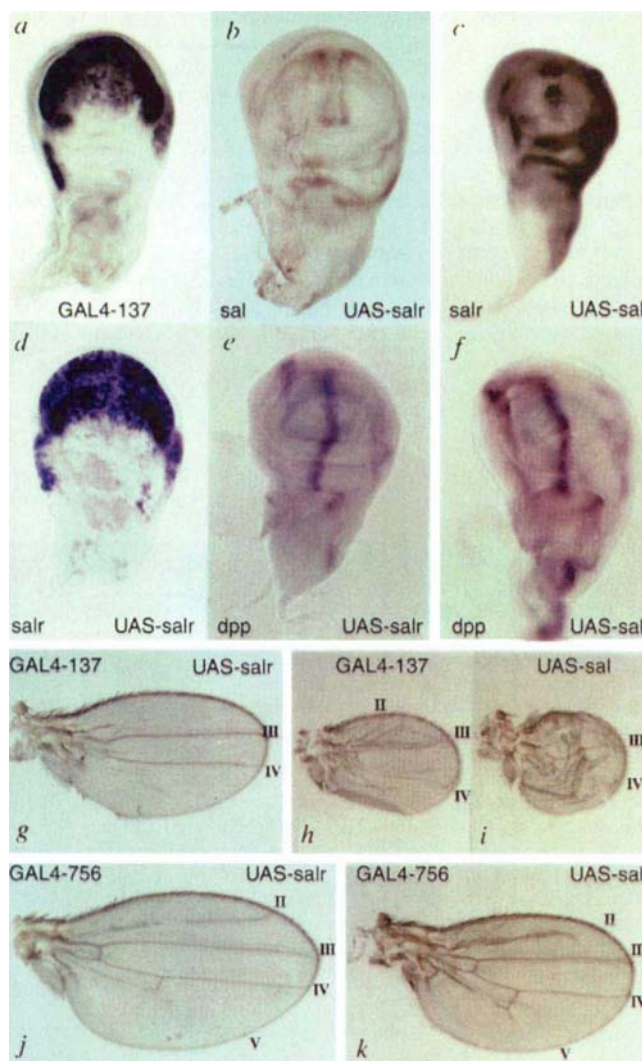


FIG. 4 Developmental consequences of *sal* and *salr* ectopic expression. *a–f*, Gene expression in experimental imaginal discs. Antibody detection of an inactive form of tetanus toxin (IMPTNT)<sup>20</sup> in the combination GAL4-137/+;UAS-IMPTNT/+ indicates that GAL4 is expressed throughout the wing blade (*a*). When *salr* is expressed using the same GAL4-137 insertion as a driver, *salr* RNA (*d*) is detected in a pattern indistinguishable from that of the GAL4 protein. In these discs the expression of *dpp* RNA is not modified (*e*), and *sal* protein expression in the wing blade is reduced (*b*). When *sal* expression is driven by GAL4-137, the disc is reduced in size, and the wing blade expression of *salr* is severely reduced (*c*). As observed with *salr*, ectopic expression of *sal* does not modify *dpp* expression (*f*). *g–i*, When GAL4-137 is used to create generalized expression of *salr* (*g*) or *sal* (*h, i*), wing size is severely reduced. The LIII and LIV veins, which normally develop within the *sal/salr* expression domain, appear in their normal positions but LII and LV are absent; some stretches of LII appear in an abnormal position (*h*), displaced to the anterior wing margin. *j, k*, When *salr* (*j*) and *sal* (*k*) are expressed using a different GAL4 line that is also homogeneously expressed in the wing blade (GAL4-756; data not shown), the phenotypes are similar but weaker and some remnants of LII and LV differentiate. METHODS. The UAS-*salr* construct was generated by cloning an EcoRI fragment from *salr* cDNA into the EcoRI site of the pUAST vector<sup>14</sup>. This fragment lacks the 3' and 5' ends of the *salr* cDNA, but contains the coding region of three groups of zinc-fingers, the putative functional domain of the protein (R.B. *et al.*, manuscript in preparation). The UAS-*sal* was made placing a BamHI-KpnI cDNA fragment containing the full *sal* open reading frame<sup>5</sup> into pUAST. After transformation using conventional techniques<sup>21</sup>, several independent lines for each transgene were established. The GAL4 lines 580 and 137 were obtained from A. Brand; GAL4-756 from J.L. Gomez-Skarmeta; and the UAS-IMPTNT from S. Sweeney. All of the combinations between GAL4 and UAS lines were analysed in double-heterozygous flies at 25 °C.

these results indicate that transcription of *sal* and *salr* is activated in response to the *dpp* protein, and raise the possibility that some effects of *dpp* in wing patterning are mediated by *sal* and *salr*. Indeed, the phenotypes shown by mosaics of *sal* and *salr* mutants (Fig. 2 and data not shown) resemble those of hypomorphic *dpp* viable alleles (Fig. 3e) and of *dpp* null alleles analysed in mosaics<sup>1</sup>; they are also similar to phenotypes produced by some viable alleles of *saxophone*, a gene encoding a *dpp*-protein receptor<sup>12,13</sup>.

The consequences of ectopic expression of *sal* and *salr*, and the possibilities of transactivation between them or feedback effects on *dpp* expression, were analysed by expanding the domain of *sal* and *salr* using the GAL4 system<sup>14</sup> (Fig. 4a, d). The ectopic expression of *sal* and *salr* throughout the presumptive wing blade dramatically affects the patterning of the wing (Fig. 4g–k). The phenotypes observed in different GAL4/UAS-*sal* or UAS-*salr* combinations include suppression or displacement of veins LII and LV associated with reductions in wing size (Fig. 4g–k). These veins normally differentiate outside the domain of *sal* and *salr* expression, and their suppression by ectopic *sal* and *salr* could result from elimination of reference boundaries between cells that express *sal* and *salr* and those that do not (see above), or from abnormal interactions between the *sal* and *salr* proteins and other putative patterning genes expressed in adjacent territories. We never observed transactivation between *sal* and *salr*; on the contrary, overexpression of *sal* reduces endogenous *salr* expression, and vice versa (Fig. 4b, c). The extent of this cross-inhibition correlates well with the reduction in wing size. The hypothesis that *dpp* acts upstream of *sal* and *salr*, and not downstream, is reinforced by the normal expression of *dpp* observed in all conditions of ectopic *sal* and *salr* expression (Fig. 4e, f). However, the ectopic expression of *sal* and *salr* does not reproduce all of the phenotypic effects of *dpp* ectopic expression<sup>4,15</sup>, indicating that additional *dpp* effector genes must exist.

It seems that in the wing disc the *dpp* protein defines a central territory of *sal* and *salr* expression that is broader than the narrow stripe of *dpp*-expressing cells. The *sal* and *salr* proteins in turn act to elaborate the growth and pattern within this territory, and affect patterning in adjacent domains, probably by establishing boundaries of gene expression. Despite obvious differences, it is tempting to speculate that the imaginal disc is subdivided into territories defined by the restricted expression of transcription factors, in a manner formally analogous to the subdivision of the *Drosophila* blastoderm into domains of gap-gene expression<sup>16</sup>. □

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# Geometric determinants of the place fields of hippocampal neurons

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THE human hippocampus has been implicated in memory<sup>1</sup>, in particular episodic<sup>2,3</sup> or declarative<sup>4</sup> memory. In rats, hippocampal lesions cause selective spatial deficits<sup>5,6</sup>, and hippocampal complex spike cells (place cells) exhibit spatially localized firing<sup>8,9</sup>, suggesting a role in spatial memory<sup>2</sup>, although broader functions have also been suggested<sup>10,11</sup>. Here we report the identification of the environmental features controlling the location and shape of the receptive fields (place fields) of the place cells. This was done by recording from the same cell in four rectangular boxes that differed solely in the length of one or both

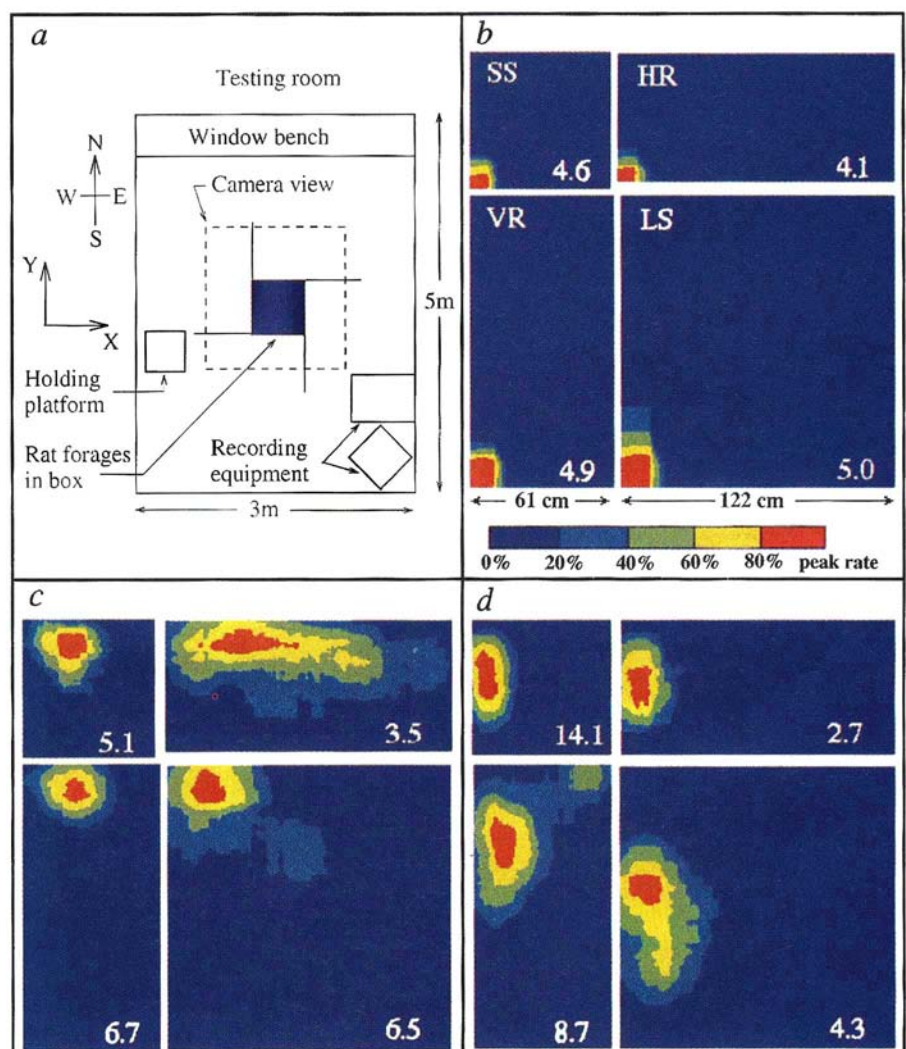
sides. Most of our results are explained by a model in which the place field is formed by the summation of gaussian tuning curves, each oriented perpendicular to a box wall and peaked at a fixed distance from it.

Place cells were recorded from six rats foraging for food in four boxes (a small square, SS; large square, LS; horizontal rectangle, HR; and vertical rectangle, VR) located within a rectangular testing room oriented north–south (Fig. 1). We first investigated whether the location of peak firing in the different boxes reflected simple determinants, such as the distance to two orthogonal box walls, or the proportion of the distance between opposite walls. Three examples of place cells that fired in all four boxes are shown in Fig. 1. The location of the place fields shows a systematic relation across the boxes. Fields B and C occur at fixed distances from the closest two walls, while field D occurs at a fixed distance from the west wall and at a fixed ratio of the distance between the north and south walls.

We analysed 28 place fields from 27 well-isolated hippocampal complex spike (pyramidal) cells (one cell had two separate fields in all boxes). Most (81 of 112) of the firing rate maps (Fig. 1) showed one contiguous region of firing with a single peak; see

FIG. 1 *a*, Plan of the testing room and the location of the camera viewing region within it. Four boxes of different shapes were set within this space: a small square (shown in blue), a horizontal rectangle, a vertical rectangle and a large square. Each box was constructed from four identical grey planks (61 cm high, 122 cm long) fastened at the top by clamps. The planks and the paper floor covering were frequently changed to ensure that local olfactory and tactile cues made no consistent contribution to cell firing. Some trials were repeated with the box in a different room location to examine the effect of room features on the location of the place field. The SS measured 61 × 61 cm, the LS 122 × 122 cm and the two rectangles 61 × 122 cm. *b–d*, Location of place field. Firing rate maps for three place cells (*b* and *c*, from region CA1; *d*, from CA3). One 8-min trial in each of the boxes (indicated by the coloured region; SS, small square; HR, horizontal rectangle; VR, vertical rectangle; LS, large square) is shown. Across all 4-boxes, the location of the peak firing rate of the cell in *b* occurs at a fixed distance from the west wall (mean 3.5 cm, s.d. 0.5 cm) and south wall ( $2.7 \pm 2.1$  cm); the peak firing rate of the cell in *c* occurs at a fixed distance from the west wall ( $24.0 \pm 6.4$  cm) and from the north wall ( $11.7 \pm 1.4$  cm), and the peak firing rate of the cell in *d* occurs at a fixed distance from the west wall ( $9.4 \pm 3.3$  cm) and at a fixed ratio of the distance between north and south walls ( $0.64 \pm 0.066$ ). These measures are referred to as the determinants of the location of the place fields; see Table 1b.

**METHODS.** Recording techniques are as described<sup>15</sup>. Briefly, 6 rats were implanted with moveable 'tetrodes' (bundles of 4 micro-electrodes) held in microdrives. Four rats were initially trained to forage in the VR, and two in the HR. During foraging the rat's location and unit activity from each tetrode were recorded. Spikes were identified by their waveform on the 4 electrodes. We selected well-isolated units, ensured that the same unit was recorded across trials by comparing waveforms, and identified recording sites as CA1 and CA3 fields of the hippocampus. Square bins 1/24 of the area of the current box were placed around grid points on the camera viewing area. The number of spikes fired by each cell and the time spent by the rat in each bin were recorded. The firing rate at each grid point is mapped as a colour plot



with linear interpolation between grid points. The 5 colours, from dark blue to red, are autoscaled so that each represents 20% of the peak rate, shown in white in the corner of each plot. Fields with a peak rate of less than 1.0 Hz are not shown.



Table 1a. The locations of the peak firing rate in most of these place fields are determined by fixed distances or proportions of distances to a box wall in the directions defined by the axes of the box (38 of 56 determinants; see Table 1b). Beyond this, 3 of the 28 cells clearly had a determinant in one direction that was fixed to some aspect of the laboratory frame (the room walls are likely candidates, as the fields moved relative to the laboratory frame in the orthogonal direction without distortion; that is, the relevant room features must be extended and parallel to the box walls).

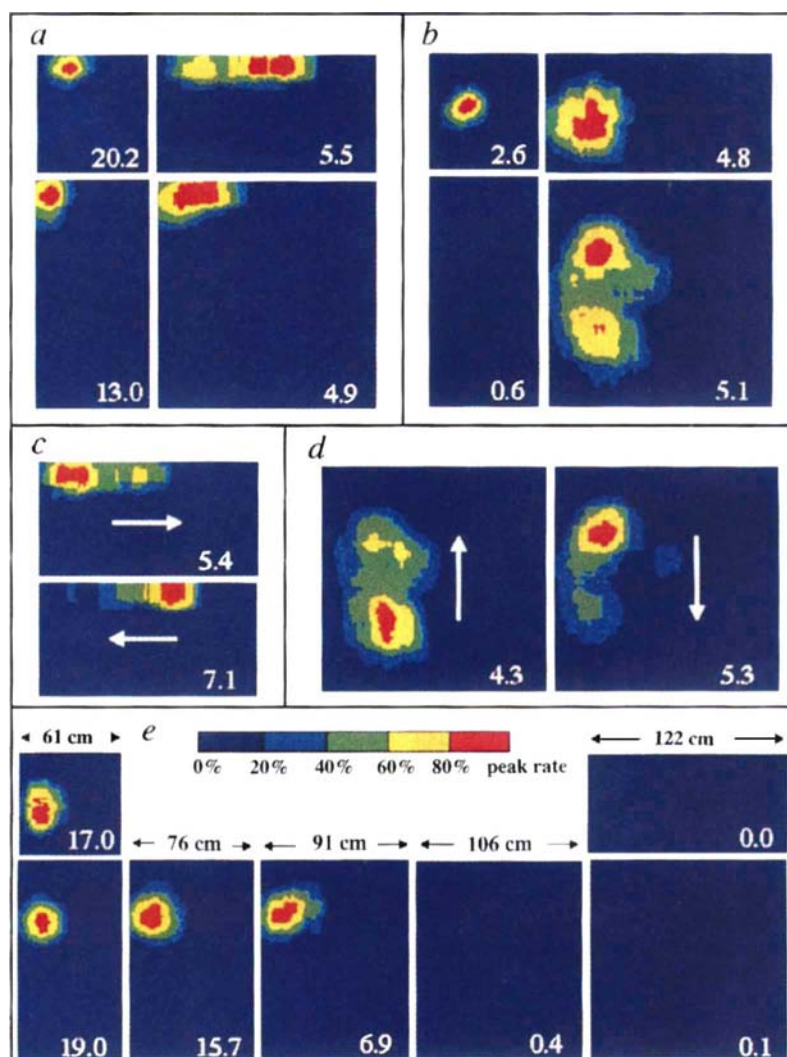
Place fields are not localized points but have two-dimensional structure. What determines their shape? For some cells, the shape of the field varied systematically between boxes differing in length along one direction: either stretching or revealing a second peak along that direction in the larger box (Table 1a). Figure 1c (SS and HR), *d* (HR and LS) shows fields stretching, and Fig. 2a (HR), *b* (LS) shows second peaks. Overall, 9 of 28 fields showed a second peak in one or more of the larger boxes. When examined, these secondary peaks accounted for some of the failures to find a determinant in the original analysis (Table 1b; Fig. 2a). The appearance of these place fields suggests a possible mechanism underlying their formation: small unitary fields might be composed of two or more independent subcomponents 'tied' at fixed distances to opposite walls; these fields then become pulled apart in one or more of the larger boxes. We examined the hypothesis that fields should be most compact in the box shape in which the animal was first trained, but found no clear evidence for this. Fields from rats initially trained in the VR, which stretch horizontally (Figs 1c and 2a), support this hypothesis, whereas fields from

rats initially trained in the VR, which stretch vertically, do not (Figs 1d and 2b).

The firing rate of place cells does not usually depend on the rat's direction in open field environments, such as cylinders and squares<sup>12,13</sup>, although it does in narrow-armed mazes<sup>12,14</sup> and linear tracks<sup>15</sup>. Many of the stretched and doubly peaked fields (22 of 24, from 14 of 15 cells) indicated a dramatic difference in the location of the peak firing rate according to the rat's direction of travel. In 13 of these (from 9 cells), the effect was straightforward: the peak of the field shifted towards the wall away from which the rat was moving. For example, if the field is stretched or split north-south (as in the LS in Fig. 2b), then when the rat is moving north the firing rate of the southern peak is much greater than when it is moving south, and vice versa for the northern peak. This effect is shown in Fig. 2c, *d* for the double fields in Fig. 2a, *b*. Our results suggest that the omnidirectional place fields seen in open field environments comprise several superimposed directional subcomponents.

We next considered the firing rates of the cells. Although these were consistent across boxes for many cells (Fig. 1b, *c*), they varied markedly for others (Fig. 2a, *b*). In some cases the peak rate fell below 1.0 Hz and no field was identified (Table 1a). Of 28 cells, 5 had fields in 2 of the 4 boxes, 9 had fields in 3, and 14 had fields in all 4. Of the 5 fields present in only 2 boxes, the boxes concerned varied in only one dimension, that is, fields never appeared solely in the SS and LS, nor solely in the HR and VR. This shows that the shape of the box *per se* was not a major determinant of the firing rate in the present experiment<sup>16,17</sup>. We hypothesized that the

FIG. 2 Dissection of the place field. *a*, *b*, Examples of how separate subcomponents of a field tied to opposing walls can sometimes be observed being pulled apart as the box expands. *a*, CA1 place cell with fields exhibiting two separate subpeaks in the HR. The determinant of its location is the distance from the north wall ( $6.7 \pm 1.8$  cm) in the Y direction and is unknown in the X direction (although if the westmost subpeak in the HR is taken the X determinant becomes the distance from the west wall). *b*, CA3 place cell that fires in three of the four boxes with a field in the LS having two separate subpeaks. The distance from the west wall ( $24.9 \pm 3.9$  cm) is the X determinant of field location, and the distance from the north wall is the Y determinant ( $35.5 \pm 6.2$  cm). *c*, *d*, Firing rate maps of *a* (HR) and *b* (LS) when the rat's direction of travel is taken into account. These maps were constructed using only the positions and spikes acquired while the rat was moving in the direction shown by the white arrow ( $\pm 90^\circ$ ). Two subcomponents of the field are revealed, with the stronger occurring earlier in the run in each case (when the rat runs away from the wall to which the location of the subcomponent is tied). Neither field was strongly modulated by the rat's direction of travel in the SS. These results imply that the small omnidirectional circular fields normally seen in open field environments may comprise superimposed directional subcomponents. *e*, This CA1 cell fired strongly in the SS and the VR, but not in the HR or LS. In further trials it was recorded in a series of boxes intermediate to the VR and LS: the cell's peak firing rate decreases monotonically with the X dimension of the box (while the Y dimension is held constant), until the rate is so low that the field disappears. Comparing locations of fields in the various boxes, including that in the SS, reveals that they remain at a fixed distance from the west wall of the box ( $15.1 \pm 0.8$  cm), and at a fixed distance from the north wall ( $36.5 \pm 4.0$  cm). These were accepted as determinants of the field location; see Table 1 legend.





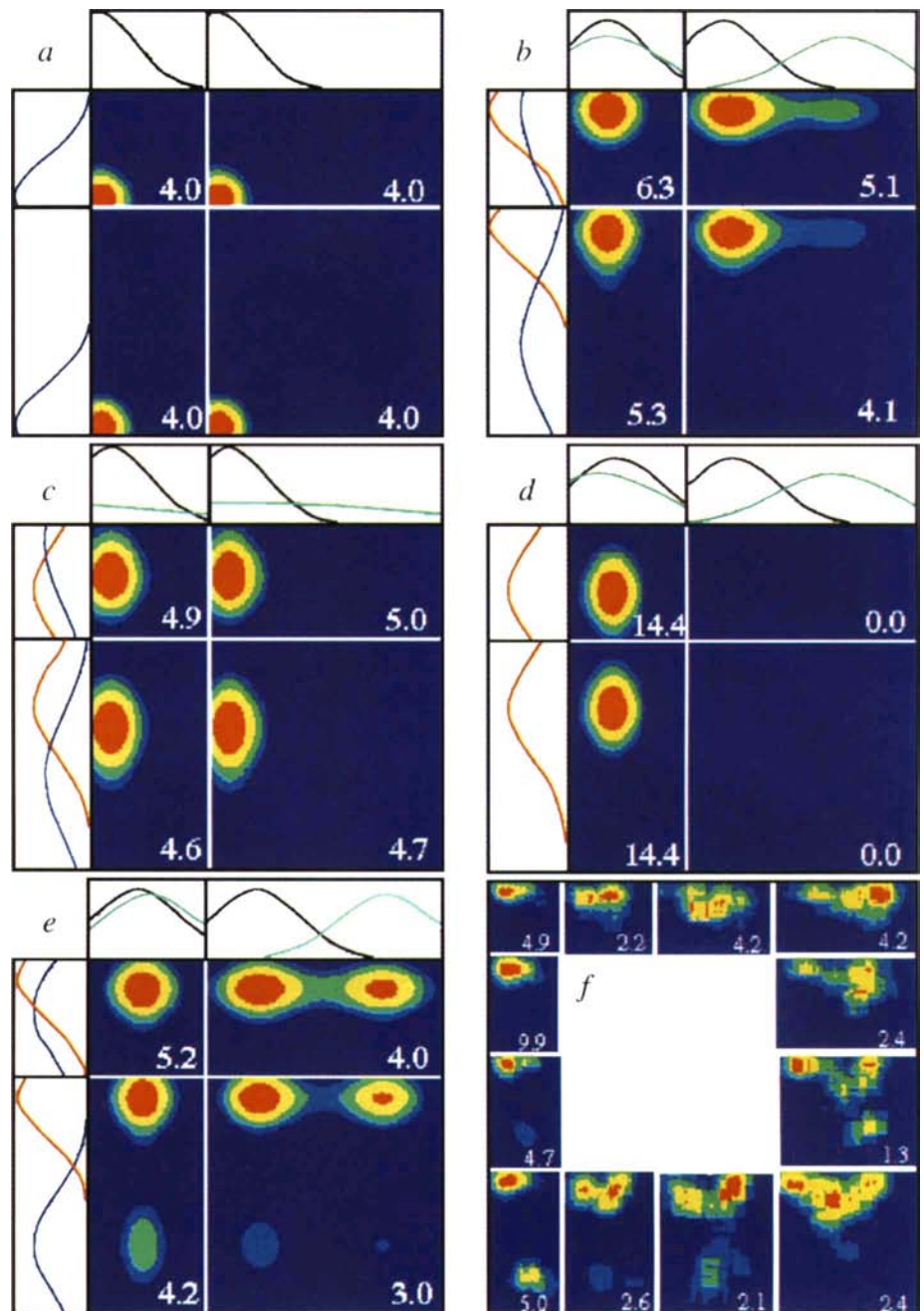
absence of firing in some boxes might be the result of a gradual decrease in firing rate as a box dimension was increased or decreased owing to a reduction in the overlap of subcomponents associated with the opposing walls. There was evidence for this in the cell shown in Fig. 2e. This cell fired at a high rate in a restricted area of the SS and the VR, but did not fire in the LS or the HR. Recording in boxes intermediate to the VR and LS indicated that the peak firing rate was controlled by the width of the box. However, this simple hypothesis does not completely explain the firing rates observed in all trials (for example, in Fig. 2b the rate in the LS should be less than in the HR).

The shape and location of the place fields found in this study can be approximately modelled as a thresholded sum of the gaussian tuning-curve functions of the distance of the rat from each box wall, where tuning curves that peak far from a wall are broader and of lower amplitude than those that peak near to a wall (see Fig. 3 legend). In general, our model assumes four gaussian functions, but in principle any number would be possible. In some cases only two or three effective inputs make for a better fit (Fig. 3a, d), and some cells appear to receive inputs fixed to the testing room as well as those from box walls (see above). We found no cells with a single input (which would resemble a stripe or an edge field).

FIG. 3 A model of place fields. The main characteristics of place field shape and location can be modelled as a thresholded sum of gaussian tuning curves in the distance of the rat from each box wall. If the four corners of a box  $w$  cm by  $h$  cm are at  $(0, 0)$   $(w, 0)$   $(0, h)$  and  $(w, h)$  then we can model a place cell's firing rate map  $f(x, y)$ , where  $(x, y)$  is the position of the rat and  $f$  is the cell's firing rate, as:

$$f(x, y) \approx A \left( \frac{\exp\{-(h-y-d_N)^2/2\sigma^2(d_N)\}}{\sqrt{2\pi\sigma^2(d_N)}} + \frac{\exp\{-(y-d_S)^2/2\sigma^2(d_S)\}}{\sqrt{2\pi\sigma^2(d_S)}} + \frac{\exp\{-(w-x-d_E)^2/2\sigma^2(d_E)\}}{\sqrt{2\pi\sigma^2(d_E)}} + \frac{\exp\{-(x-d_W)^2/2\sigma^2(d_W)\}}{\sqrt{2\pi\sigma^2(d_W)}} \right) - T,$$

where  $A$  gives the overall amplitude,  $T$  is a threshold,  $d_N$  is the value at which the tuning curve in the distance from the north wall peaks (in cm),  $d_S$ ,  $d_W$  and  $d_E$  are defined similarly, and the width  $\sigma(x)$  is an increasing function of  $x$ . Specifically, we have used  $\sigma(x) = \sigma_0(61^2 + x^2)/61^2$  so that if the rat measures the distance  $x$  to a wall as, say, the angle from vertical to the top of the wall (61 cm high), then the width of the gaussian is constant under this measure of distance ( $\sigma_0 = 20$  cm). Note that gaussians peaked close to a wall will be higher and narrower than those peaked far from one, owing to the variation in  $\sigma$ . The number of effective inputs may be made less than 4 by assigning large values to  $d_N$ ,  $d_S$ ,  $d_W$  or  $d_E$ . Colour plots show simulated firing rate maps, with the peak rate in white each corner, and the borders showing the gaussian tuning curves in the X and Y directions. a, Firing rate maps formed by summing two orthogonal gaussians in the 4 boxes. One curve is fixed to the west wall (black) and one to the south wall (blue). b, c, Summation of 4 gaussians, curves fixed to the east wall are shown in green and the north wall red. a–c, Simulations of the place fields in Fig. 1b–d, respectively, using a threshold  $T$  proportional to 80% of the peak firing rate in each box (showing the top 20% of the summed gaussian inputs in each box). d, Simulation of the cell in Fig. 2e, which fires in only 2 of the 4 boxes, using 3 inputs and a fixed threshold  $T = 75$ . Simulated fields appear in only 2 of the 4 boxes and would not appear solely in the SS and LS, or solely in the HR and VR, as in the data. e, f, Simulated and real data, respectively, in which a place field is pulled into more than 2 components as the box expands (the place cell in f was recorded in the 4 canonical boxes and 8 boxes of intermediate shape). The simulation in e used a threshold  $T$  proportional to 80% of the peak firing rate. In general, either of the above thresholding strategies (fixed across



boxes, or proportional to the peak rate) may be necessary, or even a combination of them (as Fig. 2b). We suspect that absolute firing rates (and hence the choice of threshold) reflect variables other than the geometry of the environment, as well as local interactions with other place cells. Values of the parameters ( $A$ ,  $d_N$ ,  $d_S$ ,  $d_E$ ,  $d_W$ ) were (500, 1,000, 5, 1,000, 5) for a, (500, 10, 50, 40, 20) for b, (500, 35, 50, 100, 10) for c, (2,000, 35, 1,000, 45, 25) for d, and (400, 10, 35, 30, 25) for e.

TABLE 1 Types of place field and the determinants of their location

| (a) Number and type of place fields in each box |           |        |        |        |         |
|-------------------------------------------------|-----------|--------|--------|--------|---------|
|                                                 | Box shape |        |        |        |         |
| Type of place field                             | SS        | HR     | VR     | LS     | Total   |
| None                                            | 3         | 8      | 2      | 3      | 16      |
| 1 peak (stretched)                              | 24 (1)    | 14 (2) | 23 (4) | 19 (4) | 80 (11) |
| 2 peaks                                         | 0         | 5      | 3      | 5      | 13      |
| > 2 peaks                                       | 1         | 1      | 0      | 1      | 3       |
| Total                                           | 28        | 28     | 28     | 28     | 112     |

| (b) Frequency of the determinants of location |                        |           |           |                      |         |       |
|-----------------------------------------------|------------------------|-----------|-----------|----------------------|---------|-------|
| Dimension                                     |                        | x         |           |                      |         |       |
|                                               | Determinant            | West wall | East wall | Proportion west-east | Unknown | Total |
| y                                             | South wall             | 1         | 1         | 2                    | 1       | 5     |
|                                               | North wall             | 4         | 0         | 4                    | 3       | 11    |
|                                               | Proportion south-north | 1         | 1         | 0                    | 1       | 3     |
|                                               | Unknown                | 2         | 2         | 1                    | 4       | 9     |
|                                               | Total                  | 8         | 4         | 7                    | 9       | 28    |

In a, 'None' indicates that the firing rate never exceeds 1.0 Hz; 'stretched' indicates singly peaked fields judged by eye to be at least as stretched as that in the LS in Fig. 1d; '2 peaks' indicates fields with two regions of elevated firing separated by at least half of the total width of the field (the field in the HR in Fig. 2a is an example of the minimum separation); '> 2 peaks' indicates more than two regions of elevated firing all with this separation. In b, the 'field location' in each box was the location of the peak in the firing rate map; see Fig. 1. Firing-rate maps containing no field or more than 2 peaks (see Table 1a) were excluded; the higher peak was used in those with 2 peaks. The determinant of the field location in a particular direction was defined as a measure with a standard deviation across boxes  $\sigma$  small enough to be achieved in less than 5% of Monte Carlo simulations of the measure applied to randomly placed fields, that is,  $\sigma$  is within a 95% confidence interval, equivalently  $P < 0.05$ . We examined three measures in each direction: the distance to each of the two walls perpendicular to that direction, and the proportion of the distance from one wall to the other. If more than one measure qualified as a determinant (17 of 56 cases, all a distance and a proportion for a field near to a box wall), the one corresponding to the highest confidence was taken. Determinants were scored as 'unknown' either because none of the measures were determinants (15 of 18), or because there were no fields in boxes that varied in size along the relevant direction (3 of 18). The 95% confidence interval for distances varied from  $\sigma < 2.3$  cm for a field present in two boxes to  $\sigma < 11.1$  cm for a field in all four boxes, that for proportions varied from  $\sigma < 0.018$  to  $\sigma < 0.113$ . Further simulations showed that  $P < 0.15$  for finding one or more determinants among the three measures in a direction, and  $P < 0.00001$  for finding 38 determinants of a possible 56. Where more than one trial was performed in a particular box, the firing-rate map with the highest peak rate was used for analysis and illustrations. Of the nine fields showing two peaks in one or more boxes, six originally lacked a determinant in one or more dimensions. In 4 of these, the second peak provided the missing determinant; for example, the distance from the west wall is a determinant of the field in Fig. 2a when the westmost subpeak in the HR is considered.

As the separation of two opposite walls is varied, this model predicts fields that peak at a fixed distance from a wall, fields that appear to peak at a fixed proportion of the distance between two walls, or fields that become stretched or pulled apart (using a threshold proportional to the peak rate; see Fig. 3a-c). If an absolute threshold is used across all trials, it can explain fields that disappear above or below critical values of the separation of opposing walls (Fig. 3d). In the extreme case the model predicts that a unitary field in the SS may be pulled into three or four components as the box expands, each a fixed distance from two of the walls (Fig. 3e). Data consistent with this were observed in the cell in Fig. 3f.

Place cells appear to identify environmental features such as the walls of the box on the basis of their sensory qualities and allocentric directions from the rat. Interchanging the planks that form the walls of a box, or rotating the box floor, did not alter the place field, ruling out a primary role for local odours or textures. The allocentric directions may derive from 'head-direction' cells<sup>18</sup>, which might be determined by room features or by inertial (possibly vestibular) signals<sup>19,20</sup>.

Our model builds on previous theoretical work<sup>19,21-25</sup>, but emphasizes the importance of extended cues in given allocentric directions and, in particular, the walls bounding the environment (see refs 16,17). Experimental work consistent with our model includes the demonstration that place fields can expand when box size is increased<sup>26</sup>, and that fields can be shifted by changing the location of the start box on a linear track<sup>27</sup> (or in a two-dimensional environment if the field is in or near the start box<sup>28</sup>). The directionality observed in the stretched and doubly peaked fields is consistent with an exaggerated form of the observation that place fields are more compact when each spike is considered to code for a future position of the rat<sup>29</sup>. Our results may help to explain those of previous experiments in which gerbils searched in two separate locations after two cues indicating a single reward site were pulled apart<sup>30</sup>. Finally, we have started to develop a quantitative under-

standing of how environmental features drive the firing of place cells. This should allow a more quantitative basis to be used for spatial theories of hippocampal function, and may have implications for cognitive map theory<sup>2</sup>. □

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# Associative, bidirectional modifications at the hippocampal mossy fibre–CA3 synapse

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**LONG-TERM potentiation (LTP) and long-term depression (LTD) are activity-dependent changes in synaptic strength that may serve as the cellular mechanisms of information storage in the vertebrate brain<sup>1–4</sup>. The mossy fibre–CA3 synapse displays NMDA (N-methyl-D-aspartate) receptor-independent forms of LTP<sup>5–8</sup> and LTD<sup>9–11</sup> that were thought to be non-associative<sup>9–11</sup>. Here we report that the mossy fibre–CA3 synapse displays each of the known types of LTD *in vivo*, including associative, hetero-synaptic and homosynaptic LTD. These types of LTD are induced when only two of the three conditions necessary for mossy fibre LTP induction are provided. Because some of these conditions can be provided by convergent CA3 afferents, each type of LTD can be induced in an associative manner, which suggests that LTD is involved in associative information storage. Similar to the induction of NMDA receptor-dependent LTD and LTP at other cortical synapses, mossy fibre LTD occurs when synaptic conditions are insufficient to induce LTP, and both LTP and LTD induction are influenced by previous synaptic activity, consistent with the view that common principles govern activity-dependent plasticity at cortical synapses<sup>2,12,22</sup>.**

Extracellular mossy fibre–CA3 field potentials were evoked in anaesthetized adult rats by stimulation of the mossy fibre bundle, and commissural-CA3 field potentials were evoked by stimulation of the contralateral CA3 region (Fig. 1). Associative LTP occurs when the subthreshold afferent activation for LTP induction is

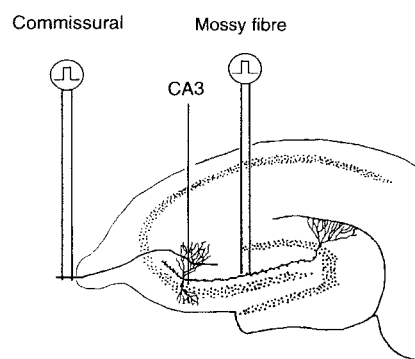
paired with high-frequency stimulation of other afferents<sup>1</sup>, but mossy fibre synapses do not show this when single mossy fibre pulses are paired with high-frequency stimulation of commissural-CA3 fibres<sup>10,13</sup>. However, the induction of mossy fibre LTP requires the activation of opioid receptors<sup>13,20</sup>. Because opioid peptides are released by the mossy fibres in a frequency-dependent manner<sup>7,13–15</sup>, associative mossy fibre LTP can be observed if  $\mu$  opioid receptor ligands are provided by high frequency mossy fibre activity<sup>13</sup>. This may confer activity-dependent constraints on mossy fibre LTP induction, limiting it to periods of repetitive granule-cell activity, for example during exploratory behaviours<sup>13</sup>. The requirement of opioid peptides for associative mossy-fibre LTP induction is demonstrated in Fig. 2a. Application of the  $\mu$  opioid-receptor-selective agonist [D-Ala<sup>2</sup>, N-Me-Phe<sup>4</sup>, Gly-ol<sup>5</sup>]-enkephalin (DAMGO) left unaltered commissural- and mossy fibre–CA3 field excitatory postsynaptic potential (EPSP) slopes, although an increase in the amplitude and number of population spikes of commissural-CA3 responses was observed, which is consistent with the disinhibitory actions of  $\mu$  opioid-receptor activation<sup>7</sup> (Fig. 2a). A single-pulse associative stimulation paradigm<sup>10,13</sup>, consisting of low-frequency mossy fibre stimulation delivered in phase with high-frequency stimulation of commissural-CA3 afferents and in the presence of DAMGO, potentiated both commissural-CA3 and mossy fibre–CA3 field EPSPs (Fig. 2a).

In contrast, single mossy fibre responses paired with high-frequency commissural stimulation in the absence of DAMGO elicit an LTD (>1 h) of mossy-fibre responses (Fig. 2b) not evident in commissural-CA3 responses. Although mossy fibre activity and coactivation with DAMGO induce mossy fibre associative LTP, in the absence of  $\mu$  opioid-receptor ligands these factors elicit in-phase<sup>3</sup> mossy fibre associative LTD, which occurs only when afferent activation is paired with stimulation of other afferents<sup>2,3</sup>.

In the absence of DAMGO, mossy fibre synaptic activity and high-frequency stimulation of commissural afferents induces associative mossy fibre LTD, but what is the effect of omitting each of the other conditions? High-frequency stimulation of commissural-CA3 afferents in the presence of DAMGO but in

FIG. 1 Schematic diagram of the *in vivo* hippocampal preparation. Field responses were recorded using a single insulated cannula placed in the stratum lucidum of the CA3 region. Mossy fibre responses were evoked by direct stimulation of the mossy fibre bundle, and commissural responses were evoked by stimulation of the contralateral CA3 region.

**METHODS.** Sixty-five adult male Sprague-Dawley rats (350–400 g) were anaesthetized with pentobarbital sodium (60 mg per kg) and maintained at 35 °C. Mossy-fibre responses were evoked by stimulation of the mossy fibre bundle in area CA3b, as described<sup>7,13,20</sup>. Commissural-CA3 responses were evoked by stimulating the contralateral CA3 region homotopic to the recording electrode. In contrast to mossy fibre responses, commissural responses are positive in stratum lucidum. Constant current monophasic pulses (30–300  $\mu$ A, 0.1 ms duration) were delivered through twisted teflon-coated stainless-steel wire, and evoked at 0.033 Hz using a current intensity that elicited responses 25% or 50% of the maximal peak amplitude. Extracellular responses were recorded using a single 33-gauge stainless-steel cannula insulated with Epoxylite, and exposed only at the tip located in the CA3a region of the dorsal hippocampus. Responses were amplified, filtered at 0.1 Hz to 10 kHz, digitized (1 kHz), stored for off-line analysis. For application of drugs to the CA3 region, the  $\mu$  opioid receptor-selective agonist DAMGO (100 pmol; RBI, Natick, MA), the  $\mu$  opioid receptor-selective antagonist Cys<sup>2</sup>, Tyr<sup>3</sup>, Orn<sup>5</sup>, Pen<sup>7</sup> amide (CTOP, 3 nmol), or lactated Ringer's were delivered by micropressure ejection<sup>7,13,20</sup>. Commissural afferents to area CA3 were tetanized using 50-ms 100-Hz bursts delivered at 5 Hz for 2 s 5 times at intervals of 30 s (refs 10, 13). In experiments in which mossy fibre stimulation was paired with high-frequency commissural stimulation, single mossy fibre responses were evoked 25 ms into each 50-ms commissural burst. In experiments in which stimulation was delivered following induction of LTD, two 1-s 100-Hz trains were delivered using current intensity that evoked mossy fibre responses that matched pre-LTD baseline responses. In experiments involving stimulation of adjacent



mossy fibres, a second electrode was placed 0.3–0.5 mm lateral to the mossy fibre-stimulating electrode. Convergence of separate mossy fibre afferents on common postsynaptic targets was verified at the end of an experiment by verifying the elimination of postsynaptic mossy fibre responses evoked by stimulation at the second electrode when delivered 5 ms after a train (100 Hz, 500 ms) to the first mossy fibre electrode. Electroencephalograms were monitored during tetanus, and animals displaying after discharges (3 of 65) were eliminated from the study. Measurement of the magnitude of CA3 responses was confined to the initial slope of the field EPSPs measured 2–4 ms after response onset. The magnitude of LTP and LTD is expressed as the percentage change in the slope of the field EPSP measured 26–30 min post-tetanus, compared with the average response amplitudes measured 5 min before application of drug or vehicle. Treatment effects were evaluated using a repeated-measures analysis of variance (ANOVA).



the absence of low-frequency mossy fibre stimulation also elicited LTD of mossy fibre responses, although commissural-CA3 responses displayed LTP (Fig. 2c). However, high-frequency commissural stimulation delivered in the presence of the lactated Ringer's medium in the absence of a  $\mu$  opioid-receptor agonist did not alter mossy fibre responses (Fig. 2d). Thus heterosynaptic mossy fibre LTD, which occurs at inactive afferents following activity of convergent afferents<sup>2,3</sup>, is observed when coactivation of commissural-CA3 afferents occurs in the presence of  $\mu$  opioid-receptor ligands and in the absence of mossy fibre synaptic activity. Although the induction of heterosynaptic mossy fibre LTP requires opioid peptides at inactive mossy fibre synapses, inactive synapses are unlikely to release opioid peptides<sup>13-15</sup>. However,

opioid peptides can exert effects far from their release sites<sup>9,14-16</sup>. Thus heterosynaptic mossy fibre LTD may normally involve diffusion of opioid peptides from neighbouring active mossy fibre synapses. The limited range of opioid-peptide diffusion<sup>16</sup> may confine this effect to diffusion-defined domains<sup>17</sup>, limiting heterosynaptic LTD to regions surrounding active synapses; this is similar to the spatial limitations of heterosynaptic LTD observed at perforant path-dentate gyrus synapses<sup>18</sup>.

We next assessed the effect of 5-Hz mossy fibre synaptic activity and the  $\mu$  opioid-receptor agonist in the absence of commissural-CA3 tetanization. DAMGO alone has no effect on mossy fibre field EPSP slopes<sup>7</sup> (Fig. 2e). However, stimulation of the mossy fibres at 5 Hz in the presence of DAMGO produced a sustained

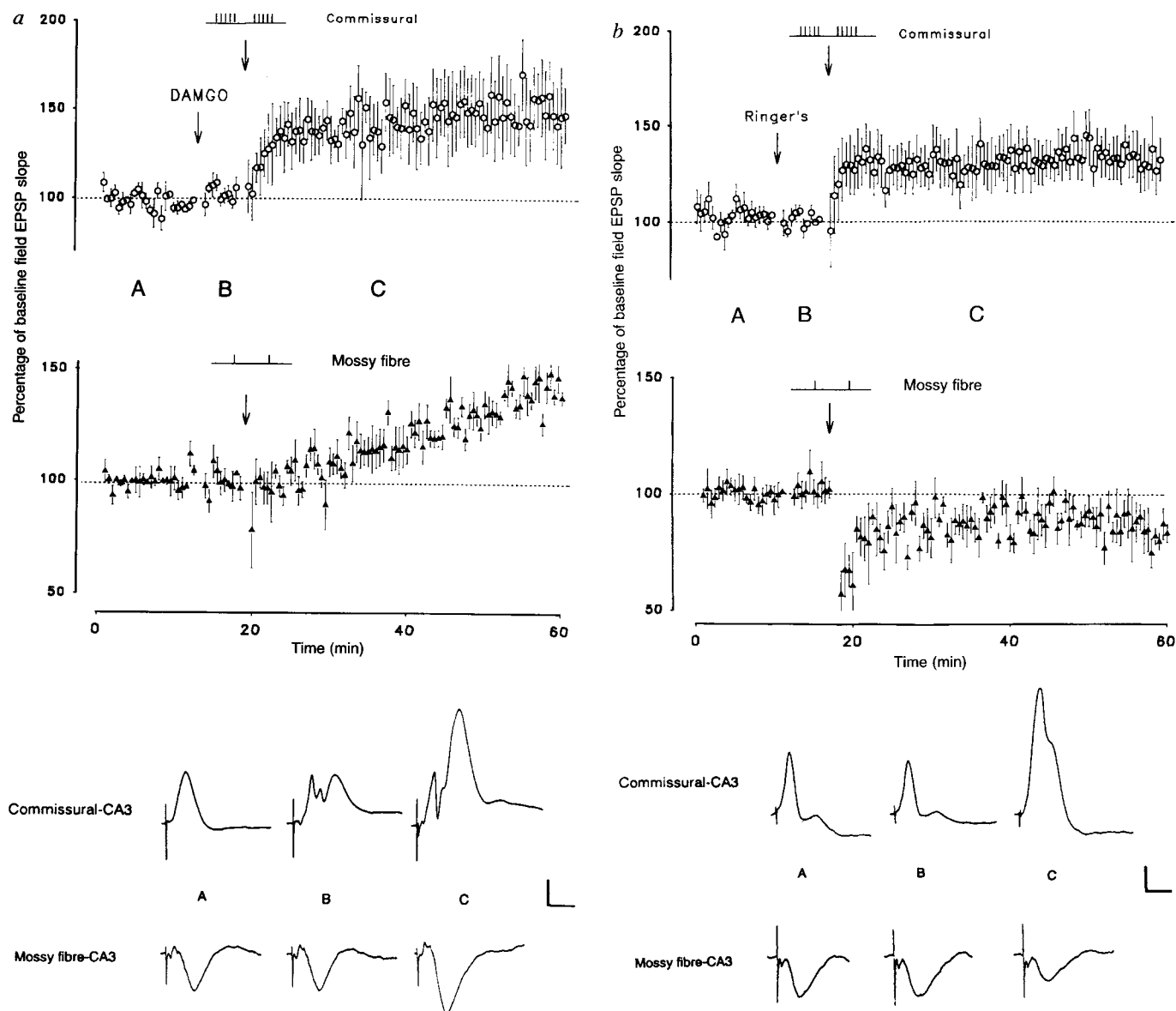


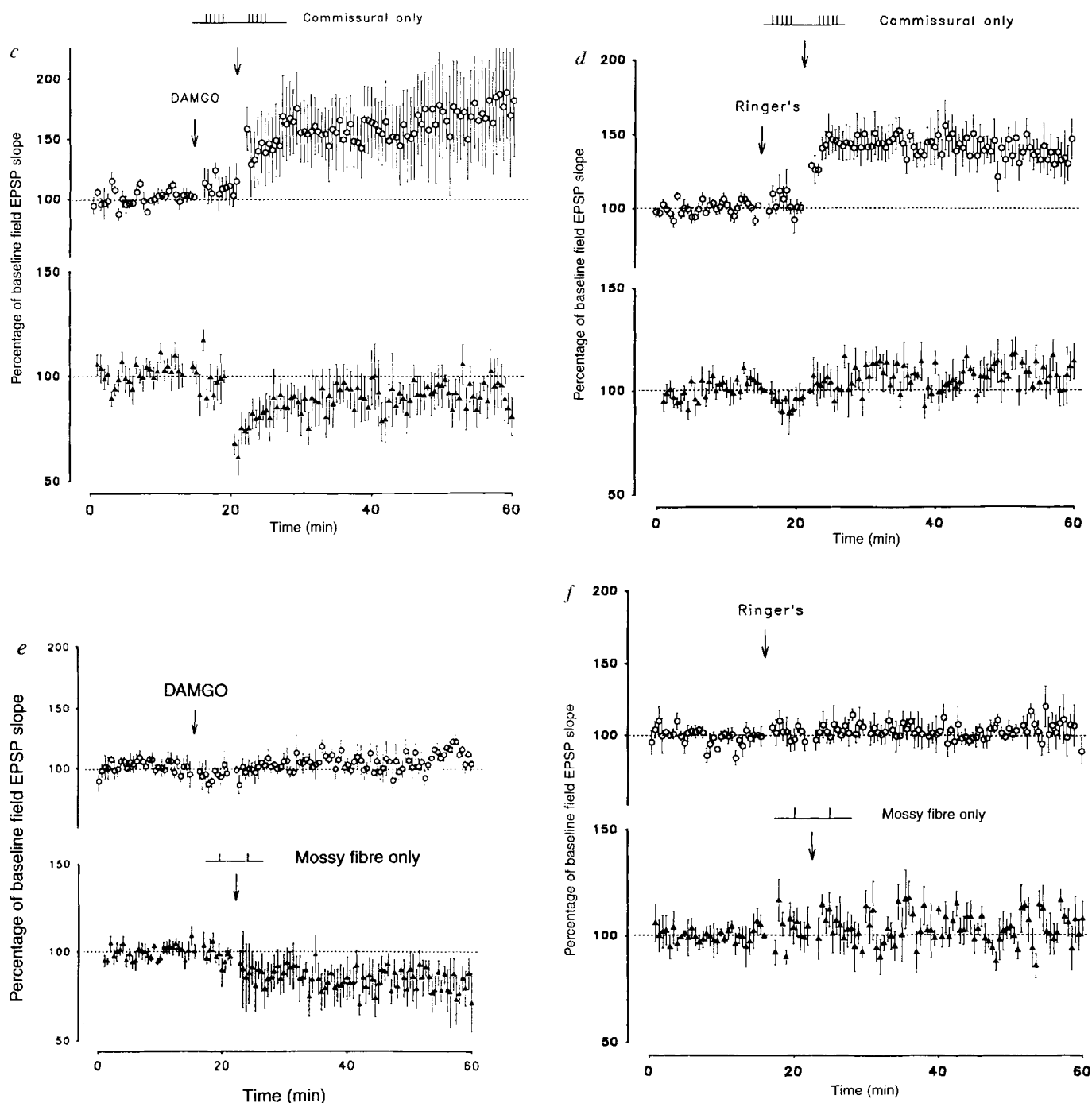
FIG. 2 a, Top, low-frequency mossy fibre stimulation (5 trains of 5 Hz, 2 s) delivered in-phase with commissural stimulation (5 trains of 100 Hz, 50 ms, delivered at 5 Hz for 2 s) and in the presence of 100 pmol of DAMGO significantly potentiated both commissural-CA3 (open circles) and mossy-fibre-CA3 (filled triangles) responses ( $F[1, 8] = 14.79$ ,  $P < 0.01$ ,  $n = 5$ ). Bottom, representative commissural-CA3 and mossy fibre-CA3 traces showing baseline responses (A), responses after application of DAMGO (B), and potentiation of mossy fibre- and commissural-CA3 responses following delivery of low-frequency mossy fibre stimulation with

commissural trains (C). Calibration: 0.5-mV, 10 ms (mossy fibre); 1 mV, 10 ms (commissural). b, Top, the stimulation parameters from a, when delivered in the presence of lactated Ringer's, produced a depression of mossy fibre responses (filled triangles,  $F[1, 14] = 7.74$ ,  $P < 0.05$ ,  $n = 7$ ), whereas commissural-CA3 responses (open circles) were potentiated. Bottom, representative traces showing baseline responses (A), responses following application of lactated Ringer's (B), and synaptic depression of mossy fibre-CA3 responses and synaptic potentiation of commissural-CA3 responses following low-frequency mossy fibre stimulation with commissural

homosynaptic depression (LTD at active synapses<sup>2,3</sup>) of mossy fibre responses (Fig. 2e). Commissural-CA3 responses were unaffected by this treatment. In contrast, a significant depression of mossy fibre responses was not observed in animals receiving 5-Hz mossy fibre stimulation in the presence of the lactated Ringer's medium (Fig. 2f). Thus homosynaptic mossy fibre LTD

is observed when mossy fibre activity and  $\mu$  opioid-receptor activation occur without convergent synaptic activity that otherwise could enable associative mossy fibre LTP induction<sup>13</sup>.

Homosynaptic LTD can be induced at other hippocampal synapses by continuous low-frequency homosynaptic stimulation<sup>10</sup>. However, we could not consistently induce mossy fibre



trains (C). Calibration: 0.5 mV, 10 ms (mossy fibre); 1.0 mV, 10 ms (commissural). c, Heterosynaptic mossy fibre LTD induced by commissural trains delivered in the presence of  $\mu$  opioid-receptor agonist. Commissural trains (5 of 100 Hz, 50 ms, delivered at 5 Hz for 2 s) delivered in the presence of 100 pmol DAMGO but without mossy fibre trains depressed mossy fibre (filled triangles) responses ( $F[1, 10] = 9.38$ ,  $P < 0.05$ ,  $n = 6$ ) and potentiated commissural-CA3 responses (open circles). d, Heterosynaptic depression of mossy fibre responses was not observed when commissural trains were delivered in the absence of DAMGO ( $F[1, 6] = 0.49$ ,

$P > 0.05$ ,  $n = 5$ ). e, Homosynaptic mossy fibre LTD induced by low-frequency mossy fibre afferent activity in the presence of a  $\mu$  opioid-receptor agonist. Low-frequency mossy fibre stimulation (5 trains of 5 Hz, 2 s) delivered in the presence of 100 pmol DAMGO but in the absence of commissural trains depressed mossy fibre (filled triangles) responses ( $F[1, 8] = 7.85$ ,  $P < 0.05$ ,  $n = 5$ ), but did not alter commissural-CA3 responses (open circles). f, LTD was not observed when low-frequency mossy fibre trains were delivered in the presence of Ringer's ( $F[1, 8] = 0.58$ ,  $P > 0.05$ ,  $n = 5$ ).

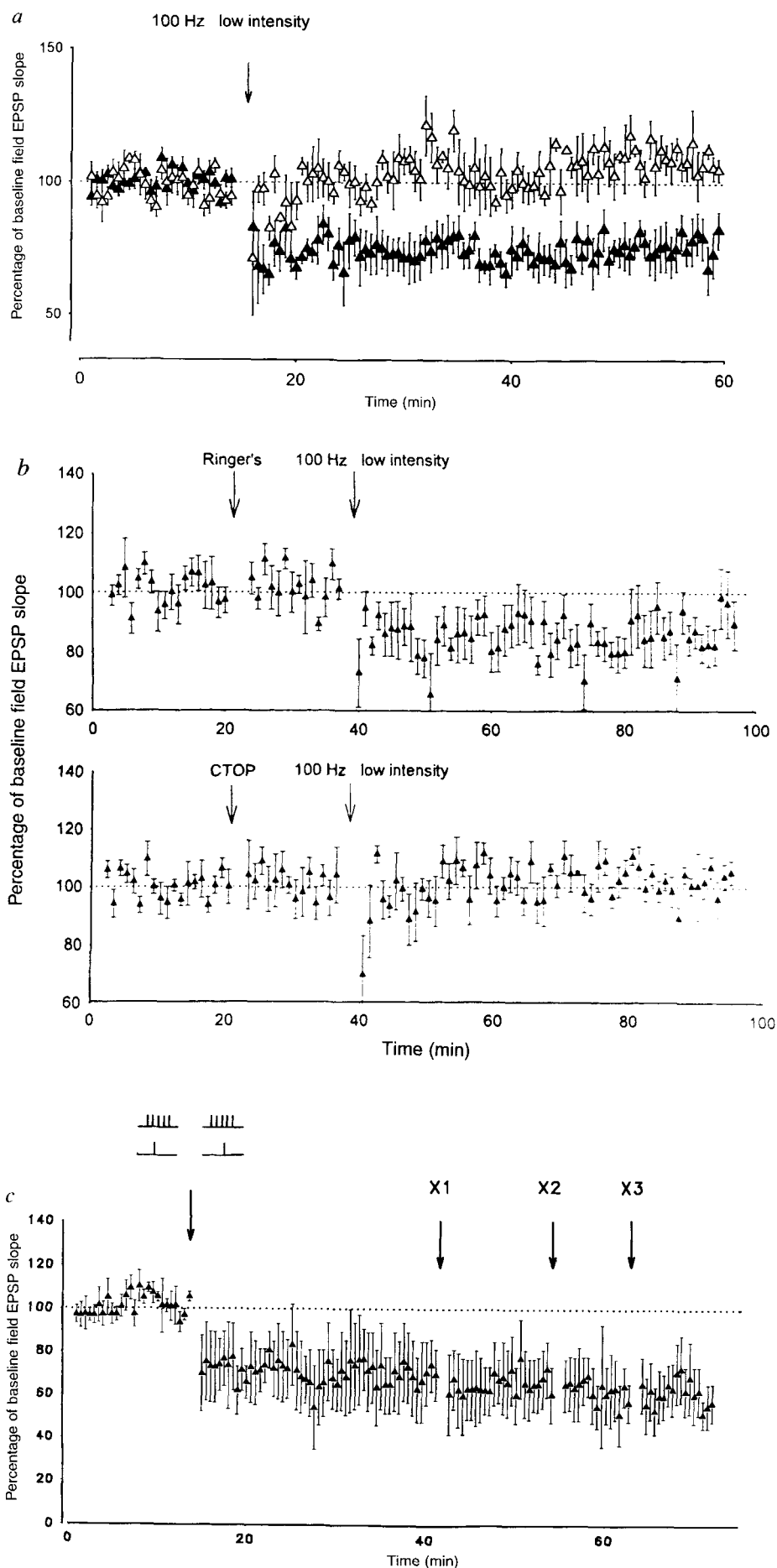
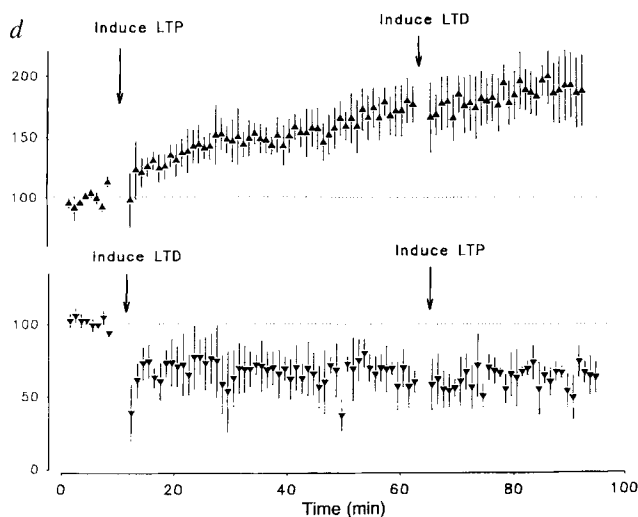


FIG. 3 *a*, Homosynaptic LTD of mossy fibre responses (filled triangles) is observed following trains (4 of 100 Hz, 0.5 s) delivered at current intensities evoking responses that were 25% of maximal amplitude ( $F[1, 3] = 12.82$ ,  $P < 0.05$ ,  $n = 4$ ). LTD is not observed in untetanized mossy fibre responses (open triangles) evoked in adjacent mossy fibres. *b*, Induction of homosynaptic LTD is blocked by 3 nmol of the opioid-receptor antagonist CTOP ( $F[1, 3] = 0.14$ ,  $P > 0.05$ ,  $n = 4$ ). Animals receiving only the lactated Ringer's displayed significant LTD ( $F[1, 4] = 8.18$ ,  $P < 0.05$ ,  $n = 5$ ). *c*, Associative mossy fibre LTD (induced as in Fig. 2*b*) reaches an asymptotic level (saturation) with repeated stimulation ( $n = 3$ ). *d*, Prior induction of mossy fibre LTP or LTD blocks subsequent induction of LTD or LTP 1 h later. Induction of LTP by high-frequency mossy fibre stimulation (first arrow, top) blocked the induction of mossy fibre LTD that is normally induced by low-frequency mossy fibre stimulation in conjunction with commissural trains (second arrow,  $n = 5$ ). Similarly, induction of associative mossy fibre LTD by low-frequency mossy fibre stimulation in conjunction with commissural trains (first arrow, bottom) blocked mossy fibre LTP normally observed following delivery of 2 trains of 1 s, 100 Hz (second arrow;  $n = 5$ ).





LTD by delivery of 900 pulses at frequencies of 1–3 Hz (data not shown). Because homosynaptic mossy fibre LTD was observed when  $\mu$  opioid-receptor activation occurred with mossy fibre activity, and because the release of endogenous opioid peptides by mossy fibre synapses requires high-frequency synaptic activity<sup>7,13–15</sup>, we determined whether homosynaptic LTD could be induced by high-frequency mossy fibre stimulation delivered at intensities insufficient to induce LTP<sup>13</sup>. Such stimulation reliably elicited homosynaptic LTD of mossy fibre responses (Fig. 3a). Homosynaptic LTD was also synapse specific, in that depression

was not observed in mossy fibre responses evoked by stimulation of adjacent mossy fibres by another electrode (Fig. 3a). Moreover, homosynaptic LTD induced by high-frequency, low-intensity mossy fibre stimulation was observed when the lactated Ringer's medium was applied to the CA3 region before delivery of low-intensity, high-frequency stimulation, whereas homosynaptic LTD was blocked by prior application of 3 nmol of the  $\mu$  opioid-receptor antagonist CTOP (Fig. 3b). Thus homosynaptic LTD following high-frequency, low-intensity mossy fibre stimulation is synapse specific and dependent on the activation of  $\mu$  opioid receptors, as is mossy fibre LTP<sup>20</sup>.

At Schaffer–CA1 synapses, repeated stimulation results in saturation of LTD<sup>21</sup>. Similarly, we observed that associative mossy fibre LTD is saturable, and that repeated stimulation produces asymptotic LTD (Fig. 3c). In addition, at Schaffer–CA1 synapses LTD can be reversed by subsequent high-frequency stimulation<sup>21</sup>. However, tetanization of mossy fibre afferents 1 h after associative LTD induction failed to either reverse mossy fibre LTD or induce mossy fibre LTP (Fig. 3d). The inability of high-frequency stimulation to reverse previously established LTD could indicate damage resulting from the stimulation parameters used to induce associative LTD. However, these same stimulation parameters were ineffective in inducing associative mossy fibre LTD when mossy fibre LTP had been induced 1 h before (Fig. 3d). Thus stimulation parameters that normally will induce LTP or LTD at naive mossy fibre synapses are ineffective at depressed or potentiated mossy fibre synapses. Together these findings suggest that mossy fibre LTD does not reflect damage to mossy fibre synapses due to the stimulation parameters; rather, induction of mossy fibre LTP or LTD appears to elicit stable potentiated or depressed states refractory to subsequent stimulation-induced alterations in synaptic responses. This effect may reflect irreversible changes in synaptic efficacy or alterations in the threshold of both LTP and LTD induction<sup>12,22,23</sup>.

Our results demonstrate that homosynaptic, heterosynaptic and

| a                          | 1 condition | 2 conditions       | 3 conditions |
|----------------------------|-------------|--------------------|--------------|
| Mossy-fibre activity       | +           | –                  |              |
| Commissural coactivation   | – no effect | +                  |              |
| Opioid-receptor activation | –           | +                  |              |
|                            |             | heterosynaptic LTD |              |
| Mossy-fibre activity       | –           | +                  | +            |
| Commissural coactivation   | +           | –                  | +            |
| Opioid-receptor activation | –           | +                  | +            |
|                            |             | homosynaptic LTD   | LTP          |
| Mossy-fibre activity       | –           | +                  |              |
| Commissural coactivation   | – no effect | +                  |              |
| Opioid-receptor activation | +           | –                  |              |
|                            |             | associative LTD    |              |

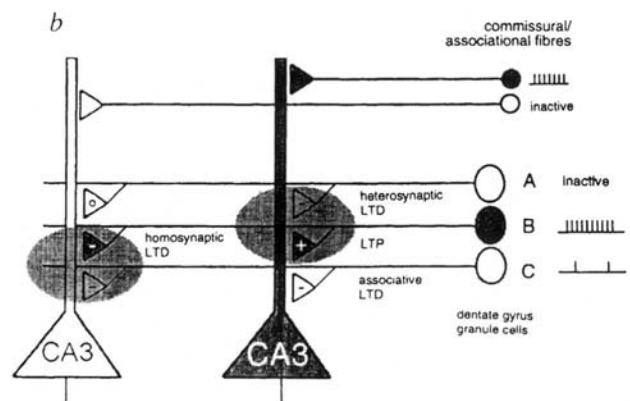


FIG. 4 Summary of the effects of combinations of the three factors essential for mossy fibre LTP induction on mossy fibre synaptic responses. a, Neither low-frequency mossy fibre activity, tetanization of commissural afferents (commissural coactivation), nor application of the  $\mu$  opioid-receptor agonist DAMGO (opioid-receptor activation) alters mossy fibre field EPSP slopes when delivered alone (1 condition). A combination of any two of these conditions elicits heterosynaptic, homosynaptic or associative mossy fibre LTD (2 conditions). A combination of all three conditions elicits mossy fibre LTP (3 conditions). b, The possible interactions of the conditions in a within the stratum lucidum of area CA3. Mossy fibre LTP is observed when repetitive granule-cell activity that is sufficient to release opioid peptides (shaded granule cell B) is coincident with convergent high-frequency commissural-CA3 activity (shaded commissural fibre) that is sufficient to depolarize CA3 pyramidal cells (shaded CA3 pyramidal cell).

Heterosynaptic mossy fibre LTD may occur at inactive mossy fibre terminals (granule cell A) that synapse on CA3 cells receiving convergent commissural-CA3 activity and that are next to peptide-releasing mossy fibre terminals (shaded regions surrounding presynaptic terminals). Associative mossy fibre LTD may occur when convergent commissural-CA3 activity (shaded commissural fibre) is coincident with mossy fibre activity that is insufficient for the release of endogenous opioid peptides (granule cell C). Homosynaptic mossy fibre LTD may occur when mossy fibre activity sufficient to release opioid peptides (shaded granule cell B) fails to activate sufficiently postsynaptic CA3 pyramidal cells. The presence or absence of convergent activity at a particular CA3 pyramidal cell could allow for a selective potentiation or depression of individual synapses along an active mossy fibre axon.

associative LTD can be observed at the mossy fibre synapse. Neither activation of  $\mu$  opioid receptors<sup>7</sup> (Fig. 2a, c, e), tetanization of commissural afferents (Fig. 2d), nor low-frequency mossy fibre afferent activity (Fig. 2f) can alone induce mossy fibre LTP or mossy fibre LTD. However, any two elicit an LTD of mossy fibre responses, and all three induce mossy fibre LTP. Because opioid-receptor ligands and sufficient coactivation can be provided heterosynaptically by neighbouring mossy fibre synapses<sup>9,14-16</sup> and by convergent CA3 afferents, respectively, other afferent systems can contribute to the induction of each type of mossy fibre LTD. Thus each type of mossy fibre LTD can be induced in an associative manner, allowing synapse-specific expression of LTP or LTD depending on the local conditions at a given synapse (Fig. 4b), and suggesting a role for mossy fibre LTD in associative information storage<sup>2-4,10,12,22,25</sup>, as is suggested for LTP<sup>14</sup>. Mechanisms of both homosynaptic and heterosynaptic LTD induction could, with LTP, increase greatly the fidelity and capacity of information storage in a sparse, distributed memory system<sup>26</sup>. Similar to LTD and LTP induction at hippocampal and neocortical synapses that are dependent on NMDA receptors, the mossy fibre synapse displays LTD when conditions approach but do not achieve those necessary to induce LTP<sup>2,12,19,22</sup>. In addition, alterations in synaptic strength are influenced by previous synaptic activity, an effect that is also observed with plasticity in both the visual cortex and area CA1 of the hippocampus<sup>23,24,27</sup>. Thus the mossy fibre-CA3 synapse displays bidirectional modifications of synaptic strength<sup>2,12,22</sup>, with the likelihood of LTP or LTD induction dependent on recent synaptic activity<sup>12,22-24</sup>. These features are characteristic of bidirectional learning rules thought to be operative at synapses in other archicortical and neocortical areas<sup>12,22,28,29</sup>. Such rules can mediate self-organization within neural networks, and theoretically can account for important computational processes such as the tuning of orientation-selective cells and the formation of the receptive fields<sup>12,22,28</sup>. The functional similarity of the rules regulating NMDA receptor-independent mossy fibre LTP and LTD induction with those at cortical synapses is consistent with the view that common principles govern activity-dependent plasticity at cortical synapses<sup>2,22,28,29</sup>. □

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## Long-term acceptance of skin and cardiac allografts after blocking CD40 and CD28 pathways

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**THE receptor–ligand pairs CD28–B7 and CD40–gp39 are essential for the initiation and amplification of T-cell-dependent immune responses<sup>1,2</sup>. CD28–B7 interactions provide ‘second signals’ necessary for optimal T-cell activation and IL-2 production<sup>3–5</sup>, whereas CD40–gp39 signals co-stimulate B-cell, macrophage, endothelial cell and T-cell activation<sup>6–12</sup>. Nonetheless, blockade of either of these pathways alone is not sufficient to permit engraftment of highly immunogenic allografts<sup>13–15</sup>. Here we report that simultaneous but not independent blockade of the CD28 and CD40 pathways effectively aborts T-cell clonal expansion *in vitro* and *in vivo*, promotes long-term survival of fully allogeneic skin grafts, and inhibits the development of chronic vascular rejection of primarily vascularized cardiac allografts. The requirement for simultaneous blockade of these pathways for effective inhibition of alloimmunity indicates that, although they are interrelated, the CD28 and CD40 pathways are critical independent regulators of T-cell-dependent immune responses.**

To explore the effect of blockade of the CD28 and CD40 pathways on T-cell proliferation, we studied primary allogeneic mixed-leukocyte reactions using T cells from C57BL/6 or L<sup>d</sup>-alloreactive (2C) T-cell-receptor (TCR) transgenic (tg) mice, as well as T cells from I<sup>k</sup>-restricted pigeon cytochrome *c*-reactive (pcc-TCRtg) mice<sup>16,17</sup>. CTLA4-Ig, an immunoglobulin fusion protein that binds to the ligands for CD28 and its homologue CTLA4 (ref. 18), effectively inhibited proliferation of all three T-cell populations (Fig. 1). In contrast, blockade of the CD40 pathway with the hamster anti-gp39 monoclonal antibody MR1 (ref. 11), moderately (~50%) inhibited the proliferation of C57BL/6 T cells responding to BALB/c dendritic cells, dramatically inhibited (≥85%) pcc-TCRtg T cells reacting to cytochrome *c* presented by I<sup>k</sup>-bearing antigen-presenting cells (APC), but had negligible effects on the proliferation of 2C T cells responding to L<sup>d</sup>-bearing BALB/c dendritic cells (Fig. 1). Furthermore, simultaneous blockade with these agents cooperatively inhibited T-cell proliferation in allogeneic mixed-leukocyte reactions and pcc-TCRtg T cells, whereas MR1 had no effect or slightly augmented the proliferation of 2C T cells when combined with CTLA4-Ig (Fig. 1). These results indicate that not all T cells are dependent on CD40 signals for clonal expansion and may explain the inability of CD40 blockade to inhibit allograft rejection completely<sup>14,15</sup>.

The effect of CD28 and CD40 blockade on T cell responses *in vivo* was assessed in C3H mice (H-2<sup>k</sup>, MMTV-7<sup>-</sup>) immunized with DBA/2 (H-2<sup>d</sup>, MMTV-7<sup>+</sup>) splenocytes in their foot pads. Five days after immunization, the draining popliteal lymph nodes in control mice treated with human IgG demonstrated a 4–6-fold increase in weight (Fig. 1c). This was accompanied by a > 30-fold expansion in the number of MMTV-7 superantigen-reactive Vβ6<sup>+</sup>CD4<sup>+</sup>T cells<sup>19</sup> (Fig. 1d) and a > 90-fold increase in the

number of  $V\beta 6^+CD4^+$  T cell blasts (Fig. 1e) within the popliteal lymph node. Alone, CTLA4-Ig or MR1 partially inhibited these responses. In contrast, the combination of CTLA4-Ig/MR1 essentially ablated the increase in lymph-node size and the expansion and blastogenesis of  $V\beta 6^+CD4^+$  T cells (Fig. 1c–e).

We next studied the effects of simultaneous treatment with MR1 and CTLA4-Ig in the vascularized murine cardiac allograft model. C3HJ recipients treated with CTLA4-Ig alone, MR1 alone, or CTLA4-Ig/MR1, all showed prolonged survival of

BALB/c ( $H-2^d$ ) cardiac allografts compared with untreated controls (Fig. 2a). However, when examined histologically at 58–62 days post-transplant, marked differences were apparent. Allografts from CTLA4-Ig-treated recipients showed extensive lymphocytic infiltration, interstitial fibrosis, and severe coronary arterial intimal thickening and fibrosis consistent with chronic rejection (Fig. 2b). Whereas MR1-treated allografts had less lymphocytic infiltration and interstitial fibrosis, these grafts also had coronary vasculopathy characteristic of chronic rejection (Fig. 2c). In marked contrast, the allografts from CTLA4-Ig/MR1-treated recipients were remarkably free from lymphocytic infiltration, fibrosis and, most significantly, coronary arterial intimal lesions (Fig. 2d). In fact, the parenchyma and blood vessels of these grafts were virtually indistinguishable from those found in normal BALB/c hearts (Fig. 2e).

Examination of T-cell cytokine and co-stimulatory molecule transcript expression in control and experimental cardiac allografts, using reverse-transcription polymerase chain reaction (RT-PCR) at eight days after transplantation, revealed that T-cell cytokine transcripts (IL-2, IL-4, IL-10 and IFN- $\gamma$ ) and co-stimulatory molecule transcripts (B7-1 and B7-2) were strongly upregulated in untreated allografts relative to normal heart tissue or syngeneic grafts (Fig. 3). CTLA4-Ig treatment of allograft recipients reduced intragraft IL-4 expression, whereas MR1 had essentially no effect on T-cell cytokine transcript expression (Fig. 3), though it partially inhibited inducible nitric oxide synthetase transcript expression, as reported<sup>15,20</sup>. Allografts from CTLA4-Ig/MR1-treated recipients showed a striking decrease in the expression of both Th1 cytokine (IL-2 and IFN- $\gamma$ ) and Th2 cytokine (IL-4 and IL-10) transcripts (Fig. 3). Interestingly, intragraft B7-1 and B7-2 co-stimulatory molecule transcripts were only moderately reduced in recipients treated with CTLA4-Ig/MR1 (Fig. 3), suggesting that CD28/B7-independent and CD40/gp39-independent factors, such as GM-CSF<sup>21</sup>, may be important regulators of intragraft B7 expression. Thus, MR1-mediated blockade of the CD40 pathway not only inhibits T-cell cognate help for B-cell, macrophage and endothelial cell activation<sup>8,9,11,12</sup>, but functionally cooperates with CTLA4-Ig to inhibit T-cell cytokine transcript expression within allografts.

To test the ability of CD40/CD28 blockade to interrupt allo-immune responses, we studied the effects of perioperative treatment with CTLA4-Ig and MR1, alone and in combination, on primary skin allograft survival in mice (Fig. 4). For comparison, recipients were also treated with cyclosporin A (CyA), an anti-CD4 monoclonal antibody YTS 191.1 (ref. 22), or with either of these agents combined with CTLA4-Ig or MR1. C3H recipients treated with either MR1, CTLA4-Ig, or YTS 191.1 alone rejected fully major histocompatibility complex (MHC)-disparate BALB/c skin grafts at essentially the same rate as untreated controls (Fig. 4a, c). Skin-graft survival was moderately prolonged in mice treated with combinations of YTS 191.1 or CyA with MR1 or CTLA4-Ig (Fig. 4b, c). However, all of these allografts were ultimately rejected. In contrast, the skin allografts on recipients treated with both MR1 and CTLA4-Ig showed markedly prolonged survival. Fifty days after transplantation, these allografts remained healthy in appearance, well vascularized, supple, and bearing short white hair (Fig. 4d). Histologically, the accepted grafts had well preserved epidermis, hair follicles and adnexal structures (Fig. 4f). Surprisingly, the salutary effect of CTLA4-Ig/MR1 on skin graft survival was abolished by concomitant CyA administration (Fig. 4b).

These data show that simultaneous blockade of the CD28 and CD40 pathways profoundly inhibits complex T-cell-dependent immune responses *in vivo*. The remarkable potency of this combination was most clearly evident in the primary skin allograft model. Neither CTLA4-Ig nor MR1, alone or in combination with either CyA or YTS 191.1, significantly prolonged skin allograft survival. Only simultaneous administration of CTLA4-Ig and MR1 produced > 50 day survival of fully MHC-mismatched skin allografts. Similar long-term survival in this stringent test of

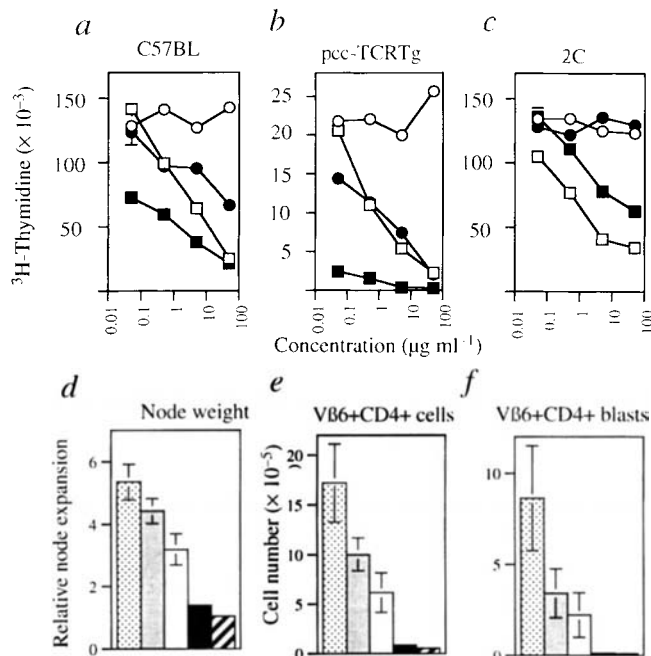
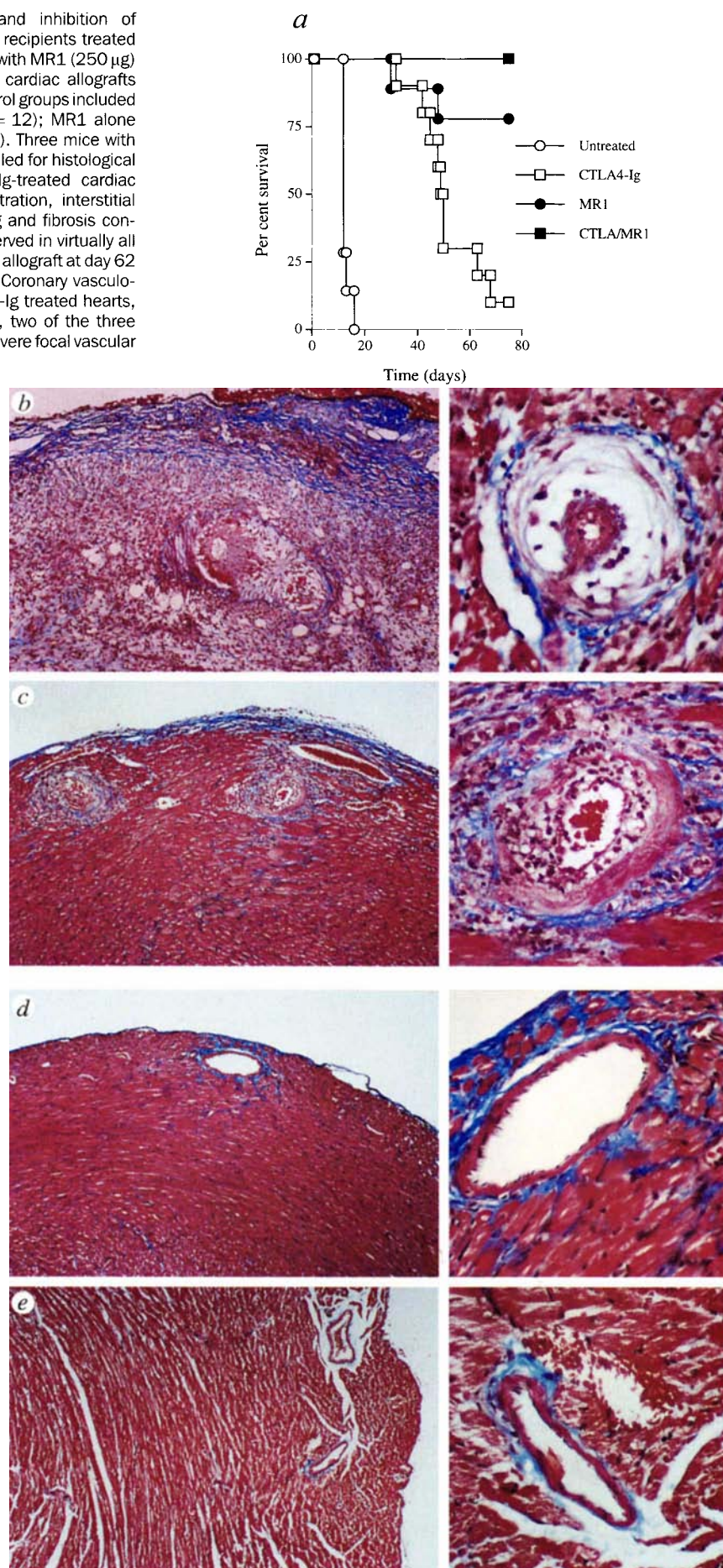


FIG. 1 a–c, Simultaneous blockade of the CD40 and CD28 pathways inhibits T-cell proliferative responses *in vitro* and *in vivo*. C57BL/6 T cells responding to BALB/c DC (a), pcc-TCRTg T cells responding to C3H splenocytes and pcc (b), or L<sup>d</sup>-alloreactive TCR transgenic T cells responding to BALB/c DC (c) were cultured with increasing doses of human Ig (○), CTLA4-Ig (□), MR1 (●) or CTLA4-Ig plus MR1 at a constant concentration of  $50 \mu g ml^{-1}$  (■). Additional controls included hamster IgG, and CTLA4-Ig titrated in the presence of hamster IgG at  $50 \mu g ml^{-1}$  (not shown). Similar results were obtained in 3 or more experiments with each cell type. d–f, Response of C3H mice to foot-pad immunization with DBA/2 splenocytes. d, Weight of the immunized popliteal lymph node relative to the contralateral node; e, number of  $V\beta 6^+CD4^+$  T cells; f, number of  $V\beta 6^+CD4^+$  T-cell blasts within the draining popliteal lymph nodes 5 d after immunization. Animals treated with human IgG (stippled), CTLA4-Ig (grey), MR1 (white), CTLA4-Ig/MR1 (black), normal unimmunized node (hatched).

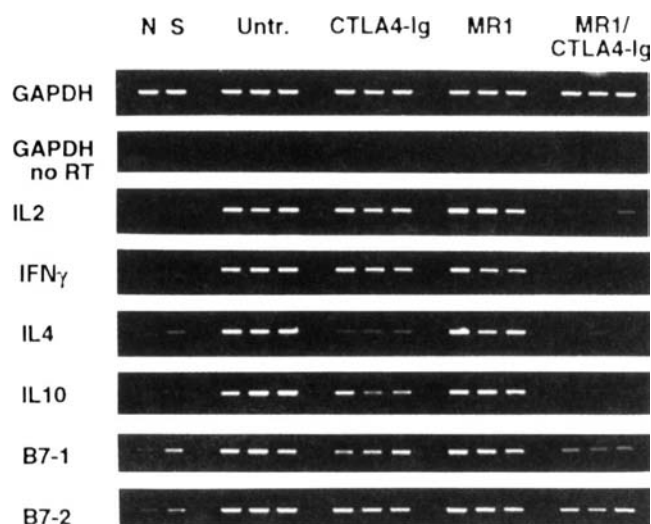
**METHODS.** a, Splenic T cells and dendritic cells (DC) were prepared as described<sup>21</sup>. T cells ( $2 \times 10^5$  per well, (a, c) or  $4 \times 10^4$  (b)) were cultured with  $2 \times 10^4$  irradiated BALB/c spleen DC (a, c) or  $(2.4 \times 10^5)$  C3H splenocytes and pcc,  $200 \mu g ml^{-1}$  (b).  $^3H$ -thymidine incorporation was measured over the last 16 h of 88- (a) or 64-h cultures (b, c). In additional experiments, proliferation was measured daily between 1 and 5 d of culture with similar findings. Male C3H (Jackson Laboratory) mice were immunized with  $2 \times 10^6$  DBA/2 splenocytes in  $50 \mu l$  sterile normal saline in the left foot pad and  $50 \mu l$  sterile normal saline in the right foot pad and then treated intraperitoneally (i.p.) with MR1 (500  $\mu g$ ), CTLA4-Ig (500  $\mu g$ ), or both reagents on days 0, 2 and 4. On day 5, the popliteal lymph nodes were removed and the weight of each node determined. Lymph node cells were counted and stained with PE-labelled anti-CD4 and biotinylated anti- $V\beta 6$  followed by FITC-conjugated streptavidin or isotype-matched controls (Pharmingen, San Diego, CA). To enumerate  $V\beta 6^+CD4^+$  T cell blasts, the forward and side scatter characteristics of the small resting population of these T cells from unprimed C3H mice were determined. Blasts were defined as cells with increased forward and side scatter characteristics relative to markers set using T cells from unprimed C3H mice. Results represent the mean of three mice per group  $\pm$  standard error of the mean.



FIG. 2 Prolongation of cardiac allograft survival and inhibition of vasculopathy associated with chronic rejection. *a*, C3H recipients treated with CTLA4-Ig (200  $\mu$ g) on days 0, 2, 4 and 6 combined with MR1 (250  $\mu$ g) on days 0, 2 and 4 had indefinite survival of BALB/c cardiac allografts (median survival time (MST) > 70 days (*d*), *n* = 7). Control groups included recipients treated with CTLA4-Ig alone (MST 50 d, *n* = 12); MR1 alone (MST 70 d, *n* = 12); or no treatment (MST 12 d, *n* = 7). Three mice with surviving transplants in each experimental group were killed for histological analysis at 58–63 days post-transplant. *b*, CTLA4-Ig-treated cardiac allograft at day 62 shows extensive lymphocytic infiltration, interstitial fibrosis, and severe coronary arterial intimal thickening and fibrosis consistent with chronic rejection. Similar findings were observed in virtually all arteries within these three grafts. *c*, MR1-treated cardiac allograft at day 62 has less lymphocytic infiltration and interstitial fibrosis. Coronary vasculopathy in MR1-treated allografts was less than in CTLA4-Ig treated hearts, with some arteries remaining well preserved. However, two of the three grafts in this group contained arteries with moderate to severe focal vascular lesions, shown in the photomicrograph. *d*, In contrast, CTLA4-Ig/MR1-treated cardiac allografts at day 58 were free of lymphocytic infiltration and fibrosis. Most significantly, coronary arterial intimal lesions were not identified in any arteries in this experimental group of cardiac allografts, nor in an additional recipient analysed 81 days after transplantation. *e*, Normal untransplanted BALB/c heart. Left and right panels were photographed at 100 $\times$  and 400 $\times$ , respectively. **METHODS.** C3H mice were transplanted with primarily vascularized BALB/c heart allografts and monitored for rejection as described<sup>15,27</sup>. Tissue sections (5  $\mu$ m) were stained with Masson's trichrome or haematoxylin–eosin. Histological specimens were reviewed by a cardiac transplant pathologist (K.J.W.) blind to the treatment modality.





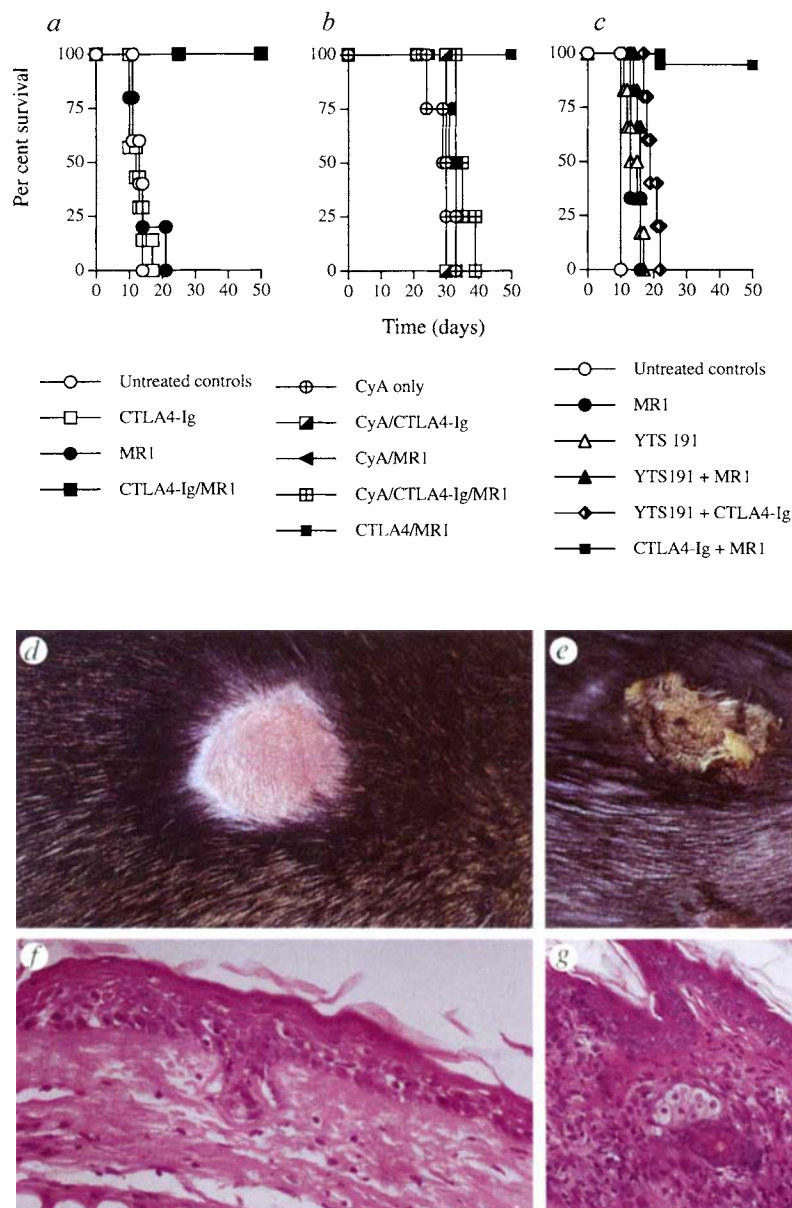


**FIG. 3** Blockade of T cell cytokine and co-stimulatory molecule transcript expression. Intragraft expression of immune mediator transcripts was assessed using RT-PCR. Three allografts from each group were analysed at 8 days post-transplant. Normal heart tissue (N) and a syngeneic heart graft (S) at 8 days after transplantation were included for comparison. Histology at this time point revealed lymphocytic infiltration in untreated controls, CTLA4-Ig and MR1 treated allografts, whereas allografts from CTLA4-Ig/MR1 treated recipients showed minimal lymphocytic infiltration (not shown). No consistent differences in the expression of T cell cytokine transcripts for IL-2, IL-4, IL-10, and IFN- $\gamma$  or co-stimulatory molecule transcripts (B7-1, and B7-2) were detectable between the control allografts (Untr.) and MR1-treated allografts, whereas CTLA4-Ig partially inhibited expression of IL-4 transcripts. Allografts from CTLA4-Ig/MR1-treated recipients showed a striking decrease in the expression of both Th1 cytokine (IL-2 and IFN- $\gamma$ ) and Th2 cytokine (IL-4 and IL-10) transcripts. However, intragraft B7-1 and B7-2 co-stimulatory molecule transcripts were only moderately reduced in recipients treated with CTLA4-Ig/MR1. PCR reactions using template prepared without reverse transcriptase yielded no products, even for the intronless GAPDH gene (GAPDH, no RT), confirming the absence of contaminating genomic DNA.

**METHODS.** At 8 days after transplantation cardiac grafts were removed. RNA preparation, cDNA synthesis, PCR and product analysis have been described<sup>15</sup>. Cycle number was optimized for each primer set to ensure that amplifications using template from the untreated allograft hearts were not at plateau (not shown). The cycle numbers for each transcript were as follows: GAPDH, 16; IL-2, 30; IL-4, 37; IL-10, 28; IFN- $\gamma$ , 23; B7-1, 29; and B7-2, 23 cycles.

**FIG. 4** Prolongation of murine skin allograft survival. C3H mice received full-thickness fully MHC-disparate skin allografts from BALB/c mice. **a**, **b**, C3H recipients treated with either MR1 alone (MST 13 d,  $n = 5$ ) or CTLA4-Ig alone (MST 12 d,  $n = 7$ ) rejected BALB/c skin grafts at the same rate as an untreated control group (MST 13 d,  $n = 5$ ). In contrast, when MR1 and CTLA4-Ig were administered together in the perioperative period, allograft survival was prolonged markedly (MST > 50 d,  $n = 15$ ) (**a**). Mice treated with CyA alone (MST 30 d,  $n = 5$ ), CyA plus CTLA4-Ig (MST 30 d,  $n = 5$ ), or CyA and MR1 (MST 32 d,  $n = 4$ ) all showed comparably modest prolonged skin graft survival. Surprisingly, the effect of CTLA4-Ig/MR1 on skin graft survival was abolished by concomitant CyA administration (MST 34 d,  $n = 4$ ) (**b**). **c**, C3H recipients of BALB/c skin grafts were not treated (MST 10 d,  $n = 3$ ), or treated with MR1 (MST 13 d,  $n = 3$ ), YTS 191.1 (MST 14 d,  $n = 6$ ), YTS 191.1 and MR1 (MST 16 d,  $n = 6$ ), YTS 191.1 and CTLA4-Ig (MST 19 d,  $n = 5$ ), or CTLA4-Ig and MR1 (MST > 50 d,  $n = 22$ ). So far, > 53 mice have been treated with CTLA4-Ig/MR1. Of these, 2 died on days 13 and 21. All others have remained healthy throughout the experiments without signs of weight loss, infection or malignancy. **d**, **e**, Healthy appearance of a BALB/c skin graft on a CTLA4-Ig/MR1-treated C3H recipient at 50 days after transplant (**d**), contrasts with a control allograft undergoing rejection (**e**). Haematoxylin-eosin-stained sections of an accepted graft 100 days after transplant demonstrates well preserved epidermis, hair follicles and adnexal structures (**f**), in contrast to a BALB/c skin graft on an untreated C3H recipient 8 days after transplant, which shows an extensive lymphocytic infiltrate (**g**).

**METHODS.** Full-thickness skin grafts (~1 cm<sup>2</sup>) were transplanted on recipient mice, secured with a Bandaid, and followed by daily visual inspection. Rejection was defined as the complete loss of visible epidermal graft tissue. Treatment protocols for MR1 and CTLA4-Ig were as for Fig. 2. CyA (Sandoz; ~20 mg kg<sup>-1</sup> d<sup>-1</sup>) was administered for 14 days via a subcutaneous Alzet osmotic pump (model no. 2002) which was implanted at the time of grafting and removed at 21 days after transplant<sup>26</sup>. YTS 191.1 (gift from K. J. Wood and A. Bushell) was administered at a dose of 150  $\mu$ g i.p. on days 0 and 1, a regimen that produces long-term acceptance of murine cardiac allografts (ref. 29 and our unpublished observations). Flow-cytometric analysis confirmed YTS 191.1 induced > 75% depletion of CD4<sup>+</sup> T cells relative to MR1 treated recipients at 23 d after transplantation (data not shown).



inhibition of the alloimmune response has previously only been observed using vigorous cytoablative and/or haematopoietic chimaerism-based strategies<sup>23–26</sup>.

These *in vitro* and *in vivo* observations suggest that although they are interrelated, the CD28 and CD40 pathways serve as independent regulators of T-cell-dependent immune responses. This is further supported by the observation that CTLA4-Ig inhibits T cell responses of gp39<sup>-/-</sup> mice, and conversely MR1 inhibits T-cell responses and allograft rejection in CD28<sup>-/-</sup> mice (our unpublished observations).

Although the molecular mechanisms remain to be determined, at a cellular level simultaneous blockade of the CD28 and CD40 pathways effectively aborts T-cell clonal expansion *in vivo* but, as evidenced by the popliteal lymph node data, does not result in clonal elimination of the responding population. The failure to

generate effective proliferative or rejection responses suggests that these cells are functionally inactivated. Furthermore, the observation that long-term acceptance of skin grafts is prevented by concomitant CyA administration suggests that inactivation of the T-cell response is an active process requiring signalling via the TcR/CD3 complex and/or other CyA-sensitive pathways.

Finally, blockade of the CD28 and CD40 pathways inhibited the development of chronic transplant vasculopathy in murine cardiac allografts. Despite the many advances in clinical immunosuppression, chronic vascular rejection remains the major source of transplant failure for which there remains no effective therapy. Further study of the functional interactions between the CD28 and CD40 pathways promises to yield new more effective strategies to manipulate T-cell-dependent immune responses, including allograft rejection. □

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## Cut2 proteolysis required for sister-chromatid separation in fission yeast

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ALTHOUGH mitotic cyclins are well-known substrates for ubiquitin-mediated proteolysis at the metaphase–anaphase transition<sup>1–4</sup>, their degradation is not essential for separation of sister chromatids<sup>5–8</sup>; several lines of evidence suggest that proteolysis of other protein(s) is required, however<sup>4,9–11</sup>. Here we report the anaphase-specific proteolysis of the *Schizosaccharomyces pombe* Cut2 protein, which is essential for sister-chromatid separation<sup>12,13</sup>. Cut2 is located in the nucleus, where it is concentrated along the short metaphase spindle. The rapid degradation of Cut2 at anaphase requires its amino-terminal region and the activity of Cut9 (ref. 14), a component of the 20S cyclosome/anaphase-promoting complex (APC), which is necessary for cyclin destruction<sup>3,4,11</sup>. Expression of non-degradable Cut2 blocks sister-chromatid separation but not cell-cycle progression. This defect can be overcome by grafting the N terminus of cyclin B onto the truncated Cut2, demonstrating that the regulated proteolysis of Cut2 is essential for sister-chromatid separation.

When *cut2-364* mutant cells were cultured at the restrictive temperature 36 °C, their phenotype resembled that previously described for *cut1* mutants, which underwent multiple rounds of the cell cycle without nuclear division<sup>13,15</sup>. Nuclear division failed

even though sister centromeres were temporarily pulled apart<sup>16</sup>. To detect Cut2 in cells, extracts from wild-type *S. pombe* were immunoblotted with affinity-purified anti-Cut2 antibodies, indicating a major band of relative molecular mass 42,000 (*M<sub>r</sub>* 42K) (see below).

More sensitive and specific detection of Cut2 in cells was accomplished by ligating the haemagglutinin (HA) epitope in frame with the C terminus of the *cut2*<sup>-</sup> open-reading frame and replacing the chromosomal copy of the *cut2* gene with the tagged version. Cut2 was localized by using the anti-HA monoclonal antibody in this strain of cells (Fig. 1). No staining was observed in the wild-type control (WT). The pattern of Cut2 immunofluorescence in the integrant changed dramatically during the cell cycle. In G2 cells that contained a single spindle-pole body (SPB), indicated by anti-Sad1 staining<sup>17</sup>, the anti-HA staining was predominantly nuclear. In mitotic cells with two SPBs separated by a small distance, the short metaphase spindle stained brightly. In sharp contrast, this staining was almost completely lost in anaphase cells with longer spindles. The nuclear staining of Cut2 was rapidly restored in post-anaphase cells, just before cytokinesis, corresponding approximately to the start of S phase.

To determine whether Cut2 was physically degraded in anaphase, we examined the Cut2 level in a *cde25* mutant strain that was blocked in G2 at 36 °C and then released at 26 °C to allow synchronous passage through mitosis<sup>18</sup>. Portions of the culture were collected at intervals after release from the block, and the level of Cut2 was examined by immunoblotting (Fig. 2*a, c*). The timing of metaphase–anaphase progression was monitored by microscopy, using DNA and anti-tubulin staining and by measurement of histone H1 kinase activity (Fig. 2*d, e*). The overall Cut2p level fell by approximately 50% during anaphase, followed by a quick recovery (Fig. 2*c*). This experiment also showed that the Cut2 band split into two (bands at 42K and 44K) upon entry into mitosis. The appearance of the upper 44K band paralleled the activity of H1 kinase and preceded the decrease in Cut2 level. The upper band was converted to the lower one by treatment with



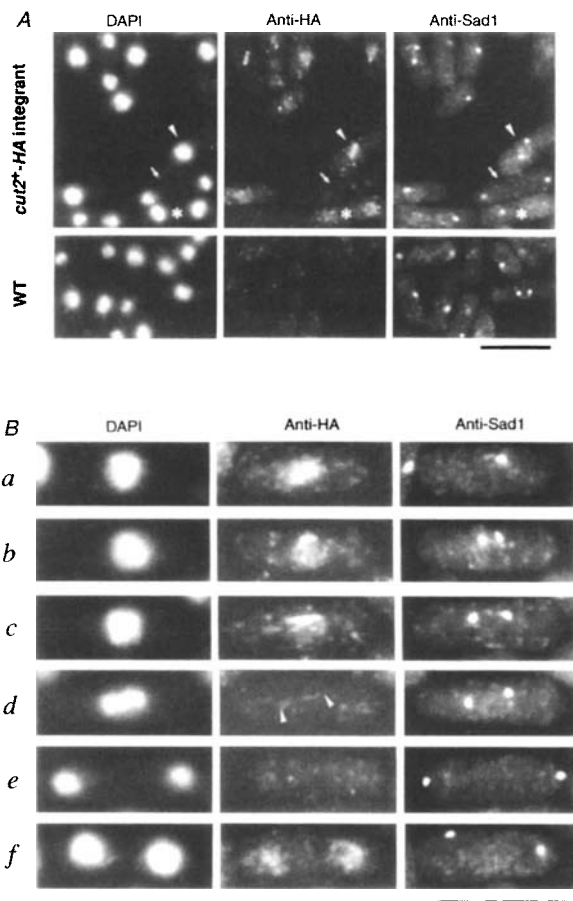


FIG. 1 Intracellular localization of Cut2. **A**, Top, HF178 (*cut2*<sup>+</sup>-HA integrant) cells stained by DAPI, anti-HA and anti-Sad1 antibodies, with metaphase (arrowhead), anaphase (small arrow) and post-anaphase cells (asterisk) indicated. Cells containing the single SPB are in G2 phase. Bottom, non-tagged wild-type cells (WT). Scale bar, 10  $\mu$ m. **B**, HF178 cells at different cell-cycle stages. **a**, G2; **b**, **c**, early to mid-mitosis; **d**, early anaphase; **e**, late anaphase; **f**, post-anaphase before cytokinesis. Arrowheads indicate SPBs. Scale bar, 10  $\mu$ m. **METHODS.** Immunofluorescence procedures using glutaraldehyde and paraformaldehyde fixation are as described<sup>24</sup>. SPBs were stained by rabbit polyclonal anti-Sad1 antibodies and fluorescein isothiocyanate conjugated anti-rabbit IgG antibodies (Cappel). HA epitopes are stained by 12CA5 (BabCO) and CY3 conjugated anti-mouse IgG (Chemicon), chromosomal DNA by 0.2  $\mu$ g ml<sup>-1</sup> DAPI. For epitope tagging of the *cut2*<sup>+</sup> gene, the C terminus of the *cut2*<sup>+</sup> genes was ligated to the triple tandem HA1 epitope<sup>25</sup>. The plasmid pHAC21, consisting of a 520-bp C-terminal fragment of the *cut2*<sup>+</sup> gene and the triple HA1 epitope followed by the *nmt1*<sup>+</sup> 3'-non-coding region and the *S. cerevisiae* LEU2 gene in pBluescript KS(+), was used for transformation of the strain *h*<sup>-</sup> *leu1*-32 *cut2*-364 at 26 °C. A stable *Leu*<sup>+</sup> strain at 26 °C and 36 °C was designated HF178, and verified to be the *cut2*<sup>+</sup>-HA integrant.

alkaline phosphatase, indicating that the 44K band represents a phosphorylated form of Cut2 (not shown). The level of both bands fell in parallel in anaphase, whereas the level of *cut2*<sup>+</sup> messenger RNA was not significantly reduced during mitosis (Fig. 24b). We therefore conclude that at least half of the Cut2 was degraded as cells entered anaphase. These results indicate that the level of Cut2 oscillates during the cell cycle, very much like cyclin B.

Proteolysis of Clb2, a mitotic B-type cyclin of *Saccharomyces cerevisiae*, begins at the onset of anaphase and continues throughout G1 (ref. 19). We therefore examined the stability of Cut2 in cells arrested in G1 or G2 by pulse-chase labelling experiments using [<sup>35</sup>S]methionine (Fig. 2B). Cut2 was expressed from the weakly inducible *nmt1* promoter of pREP81 (ref. 20) in cells

arrested in G1 (*cdc10*)<sup>21</sup> or G2 (*cdc25*) cells. Quantification of the levels of labelled Cut2 in this experiment showed that the half-life of the protein declined from 16 min in cells at the *cdc25* arrest point to 2 min in cells at the *cdc10* arrest point, indicating that the stability of the protein declines sharply at the end of mitosis.

We investigated whether Cut2 degradation was dependent on ubiquitin-mediated proteolysis promoted by the 20S cyclosome/APC. In *S. pombe*, Cut9 is the homologue of *S. cerevisiae* Cdc16, one of the components of the 20S complex<sup>4,14</sup>. We constructed a double *cdc10*-*cut9* mutant, and examined the stability of Cut2 in the G1-arrested double mutant. Wild-type, *cdc10* and *cdc10*-*cut9* cells were arrested in G1 by nitrogen starvation at the permissive temperature<sup>22</sup>. These cultures were then shifted to the restrictive temperature for 0–6 h in the presence of a nitrogen source. This treatment allowed growth to resume, but cells carrying the *cdc10* mutation remained arrested in G1. In all three strains, the level of Cut2 mRNA increased to similar levels after release from nitrogen starvation (Fig. 2C, bottom). The Cut2 level increased in wild-type cells (Fig. 2C, top right), but did not in the *cdc10* mutant, as expected from the pulse-chase data (Fig. 2C, top left). In the *cdc10*-*cut9* double mutant, however, the Cut2 level strongly increased (Fig. 2C, top middle), although these cells were still arrested in G1, as judged by their 1C DNA content. These data indicate that the *cut9*<sup>+</sup> gene is required for the degradation of Cut2.

The N-terminal portion of mitotic cyclins is not necessary for binding to and activation of p34<sup>cdc2</sup>, and is solely required for mitotic destruction<sup>23</sup>. This region contains an amino-acid motif called the destruction box, which is required for regulated proteolysis<sup>1</sup>. The N-terminal region of Cut2 contains two sequences with similarity to mitotic cyclin destruction boxes (Fig. 3a). To test whether these putative destruction boxes in Cut2 regulate protein stability or sister-chromatid linkage, we constructed a conditionally expressed truncated *cut2* mutant. A version of Cut2 lacking the N-terminal 80 amino acids was expressed from the weak *nmt1* promoter of pREP81, which can be induced by the removal of thiamine from the medium. This truncated protein (Cut2Δ80, with an apparent mobility of 37K) accumulated to high levels in G1-arrested cells (Fig. 3b), unlike wild-type Cut2, which was undetectable even though the mRNA was present at the same level in these conditions as in the asynchronous or G2-arrested cells (Fig. 3b). The DNA content remained at 1C (data not shown), and the level of Cdc13 (*S. pombe* cyclin B, indicated by p63) was extremely low in G1-arrested cells, so production of Cut2Δ80 did not overcome the *cdc10* G1 arrest.

We next investigated the effect of non-degradable Cut2Δ80 on cell division. The expression of Cut2Δ80 from the weak REP81 promoter blocked colony formation, whereas expression of wild-type Cut2 under the same promoter did not (Fig. 4c, top). In liquid culture, production of the plasmid-encoded proteins could easily be detected 12 h after the removal of thiamine at 33 °C (data not shown). At this time, the proportion of cells displaying a pair of separated daughter nuclei decreased dramatically (from 16% to 3.7%) in the strain producing Cut2Δ80, but remained high (16%) in the strain producing wild-type Cut2. *In situ* hybridization with a centromeric probe confirmed that sister-chromatid separation was defective in cells producing truncated Cut2Δ80, even though the spindle formed and chromosomes condensed normally (Fig. 3c, right). In contrast, normal sister-chromatid separation was found in cells producing wild-type Cut2 (Fig. 3c, left). Cells producing Cut2Δ80 continued to progress through the cell cycle in the absence of sister-chromatid separation, resulting in the appearance of the cut phenotype<sup>12</sup> (43% of cells at 14 h), which was much less frequent (5%) in cells producing wild-type Cut2.

We wished to test whether the function of the N-terminal region of Cut2 is to target the protein for destruction at the onset of anaphase. If this hypothesis is correct, it might be possible to rescue the lethal phenotype of Cut2Δ80 by grafting on the N-terminal region of a mitotic cyclin containing the mitotic destruction signal. Chimaeric plasmids were constructed in

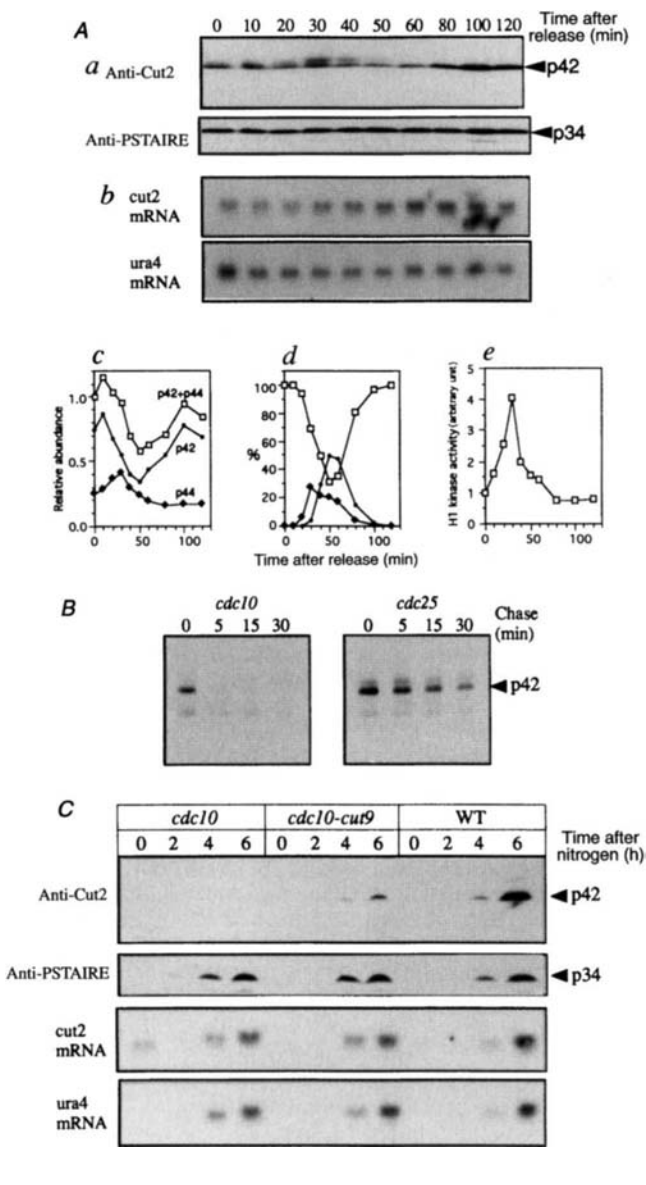
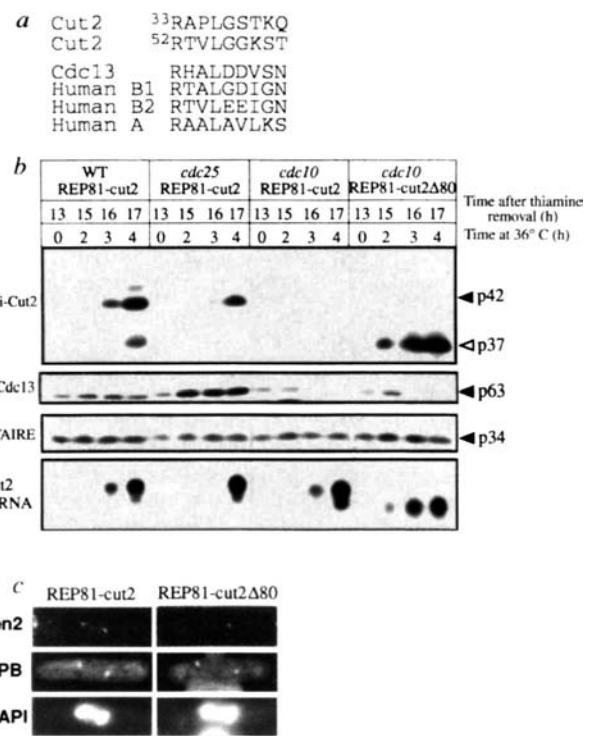


FIG. 2 Cut2 is degraded at anaphase and in G1-arrested cells. **A**, Ts *cdc25-22* mutant cells blocked in G2 at 36 °C for 4 h were released at 26 °C. **a**, Cut2 level was determined by immunoblotting using anti-Cut2 antibodies; anti-PSTAIRE was a loading control. **b**, Levels of *cut2*<sup>+</sup> and *ura4*<sup>+</sup> mRNA (northern blot). **c**, Quantitative analysis of 42K and 44K band intensities. **d**, Frequency of interphase (open squares), metaphase (filled diamonds) and anaphase (small circles) cells determined by DAPI and tubulin staining. **e**, Histone H1 kinase activity of extracts. **B**, Pulse-chase labelling of Cut2. G2-arrested *cdc25-22* cells and G1-arrested *cdc10-129* cells were labelled with [<sup>35</sup>S]methionine for 5 min at 36 °C and chased by excess cold methionine and cysteine for 0, 5, 15 and 30 min. Immunoprecipitates using anti-Cut2 antibodies were analysed by SDS-PAGE, followed by fluorography. **C**, Wild-type, *cdc10* and *cdc10-cut9* double mutant strains were starved for nitrogen at 26 °C, and the resulting cells containing 1C DNA content were cultured at 36 °C for 0–6 h in with a nitrogen source. Cut2 and Cdc2 levels, and *cut2*<sup>+</sup> and *ura4*<sup>+</sup> mRNAs (per cell), were examined by immunoblot and northern blot, respectively.

**METHODS.** Bacterially expressed Cut2 was used for raising rabbit polyclonal antibodies. Yeast cells were broken in lysis buffer containing 50 mM Tris-HCl, pH 7.5, 10 mM EDTA, 10% glycerol, 10 mM *N*-ethylmaleimide and protease inhibitors (1 mM PMSF, 2 μg ml<sup>-1</sup> pepstatin A and 10 μM E-64). The supernatant after low-speed centrifugation (1,500g, 5 min) was used for immunoblotting. For quantification of Cut2, the intensity of chemiluminescence (ECL, Amersham) was estimated by a GS-250 Molecular Imager (Biorad). Intensities were calibrated using 0.25-, 0.5- and 1.5-fold amount of 0 time-point extracts. Intensities of anti-PSTAIRE bands were used as a loading control. For pulse-chase experiments, the procedures for *S. cerevisiae*<sup>26</sup> were modified. The *cdc10* or *cdc25* strain carrying pRECUT2-5, which overproduced Cut2 from REP81 promoter<sup>20</sup>, were incubated in 80 ml EMM2 medium at 26 °C for 14.5 h after thiamine removal. After a 3.5 h shift to 36 °C, cells were concentrated in 5 ml sulphate-free EMM2 to 5 A<sub>600</sub> units per ml. After 5 min preincubation at 36 °C, 2 mCi Tran<sup>35</sup>S-label (ICN) was added. After 5 min labelling at 36 °C, the culture was transferred to 80 ml EMM2 containing 5 mM methionine, 2 mM cysteine and 2 μM thiamine at 36 °C. Portions of 19 ml were removed when indicated and mixed with an equal volume of ice-cold 20 mM Na<sub>3</sub>EDTA.

FIG. 3 Non-degradable Cut2 blocks sister-chromatid separation. **a**, Comparison of the potential destruction boxes in Cut2 with the destruction boxes of Cdc13, human cyclin B1, B2 and A. **b**, Wild-type *cut2*<sup>+</sup> under the weak *nmt1* promoter (pREP81) was induced (REP81-cut2) in wild-type, *cdc10* and *cdc25* mutant strains. The mutant *cut2Δ80* under the same promoter was also induced (REP81-cut2Δ80) in *cdc10* mutant. Cells were collected 13, 15, 16 and 17 h after thiamine removal. These cultures were transferred from 26 °C to 36 °C after 13 h (0 h at 36 °C). Results of immunoblot using anti-Cut2, anti-Cdc13, anti-PSTAIRE antibodies and northern blot using the *cut2Δ80* gene as a probe are shown (p37 is Cut2Δ80p). **c**, Induction of wild-type Cut2 or Cut2Δ80 from REP81 promoter in wild-type cells. The cells were cultured for 13 h at 33 °C after thiamine removal. Wild-type cells expressing Cut2 (left) or Cut2Δ80 (right) were observed by fluorescence *in situ* hybridization using a unique *cen2* probe (cos 1228)<sup>27</sup>. Scale bar, 10 μm.



which the N-terminal 77 residues of Cdc13 were fused to the N terminus of Cut2Δ80. Cdc13N–Cut2Δ80 contained the wild-type destruction signal, whereas Cdc13N\*–Cut2Δ80 carried a mutation in the destruction box (Fig. 4a). The chimaera containing the wild-type Cdc13 destruction box was unstable in G1-arrested cells (Fig. 4b) like Cut2 itself, whereas the indestructible mutant chimaera Cdc13N\*–Cut2Δ80 accumulated strongly in G1-arrested cells, like Cut2Δ80. This result shows that the N terminus of Cdc13 could confer G1 instability on Cut2Δ80.

Expression of either Cut2Δ80 or the indestructible chimaera Cdc13N\*–Cut2Δ80 in a wild-type strain under the REP81 promoter strongly inhibited colony formation (Fig. 4c, top). In contrast, expression of the destructible chimaera Cdc13N–Cut2Δ80 allowed colonies to form (Fig. 4c, top). The destruction signal of Cdc13 was therefore able to suppress the toxicity of Cut2Δ80. Most strikingly, the destructible chimaera Cdc13N–Cut2Δ80 was able to complement the temperature sensitivity of

*cut2*–364, whereas the indestructible chimaera Cdc13N\*–Cut2Δ80 could not (Fig. 4c, bottom). Thus addition of the N-terminal 77 residues of Cdc13 to the truncated Cut2 allowed a previously toxic protein to support normal sister-chromatid separation, although there is virtually no sequence similarity between Cut2 and Cdc13, apart from their destruction boxes. These data demonstrate that the regulated proteolysis of Cut2 is necessary for sister-chromatid separation. Point-mutation analysis indicates that both destruction-box sequences in Cut2 (Fig. 3a) are functional *in vivo* (unpublished data).

Our data show that Cut2 is an excellent candidate for a protein whose degradation is required for sister-chromatid separation. They also suggest that Cut2 and mitotic cyclins are destroyed by the same ubiquitin-dependent destruction machinery including the cyclosome/APC<sup>3,4,11</sup>. The expression of non-degradable cyclin B, which prevents the inactivation of p34<sup>cdc2</sup> and cell division, does not block sister-chromatid separation<sup>7–8</sup>. In contrast, the expression of indestructible Cut2 blocks sister-chromatid separation but does not prevent the inactivation of p34<sup>cdc2</sup> and cell division. These results indicate that the degradation of cyclin B and Cut2 play complementary roles in the exit from mitosis. If Cut2 functions as an inhibitor of premature sister-chromatid separation, null mutants would be expected to show a phenotype of the separation of sister chromatids before mitosis. Surprisingly, *cut2Δ* mutants have a phenotype very similar to that of the non-degradable *cut2Δ80* mutant (data not shown). The simplest explanation of this apparent paradox is that Cut2 is involved in two sequential functions that are required for sister-chromatid separation. The first, which remains hypothetical, is to convert the sister chromatids of G2 cells into a form in which they can be separated from one another. The second would be to prevent sister-chromatid separation from occurring until the activation of the cyclin proteolysis machinery. Cut2 and Cut1 (ref. 13) associate together in a high-molecular-weight complex (unpublished data), and we suspect that Cut2 regulates Cut1. One possibility is that Cut1 is primed for activity by association with Cut2, but the final activation of Cut1 to separate sister chromatids requires the destruction of Cut2. Further research on the role of the Cut1/Cut2 complex in sister-chromatid separation is needed to reveal the molecular mechanisms that allow a protein simultaneously to potentiate and inhibit critical cell-cycle events. □

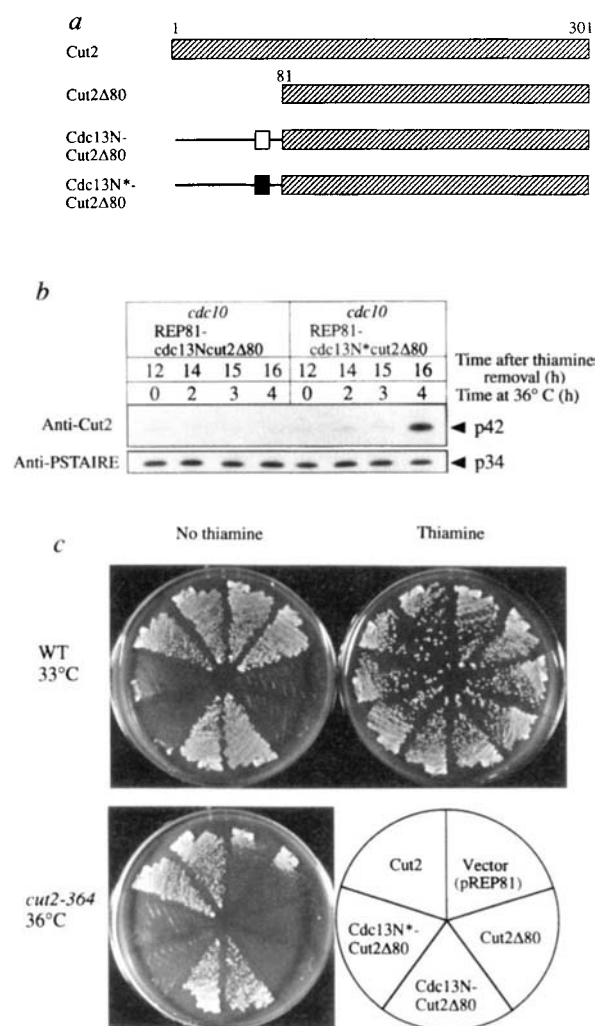


FIG. 4 Effects of Cdc13 destruction signal on Cut2Δ80. **a**, Full length Cut2, Cut2Δ80, Cdc13N–Cut2Δ80 chimaera, which is the fusion of the N-terminal 77 amino acids of Cdc13 and Cut2Δ80, and Cdc13N\*–Cut2Δ80, which is the fusion of the N-terminal 77 amino acids of Cdc13 whose destruction box was mutated from R<sup>59</sup>HALDDVSN to A<sup>59</sup>HAADDVSN and Cut2Δ80. **b**, Immunoblot patterns using anti-Cut2 antibodies. Left, extracts of *cdc10* mutant expressing the chimaera gene *cdc13N* and *cut2Δ80* at 36°C under the REP81 promoter. Right, extracts of *cdc10* producing the mutant chimaera gene *cdc13N\** and *cut2Δ80*. **c**, Wild-type (top) or *ts cut2*–364 (bottom) strains carrying the plasmids that have variants of *cut2* genes under the REP81 promoter were incubated in the absence or presence (2 μM) of thiamine. Wild-type and *cut2*–364 strains were incubated at 33°C and 36°C, respectively.

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# Ribozyme-catalysed amino-acid transfer reactions

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THE 'RNA world' hypothesis proposes an early stage in the evolution of life in which both genomic and catalytic functions were fulfilled by RNA<sup>1</sup>. The evolution of RNA-catalysed protein synthesis would have been a necessary step in the transition from such an RNA world to modern protein-dominated biology. For this to have been possible, RNA must be capable of catalysing amide-bond formation using acylated carrier RNA substrates as amino-acid donors. We have used *in vitro* selection and evolution to isolate ribozymes with acyl transferase activity from a pool of random RNA sequences. One of these acyl transferases with a 5'-amino group transfers an amino acid to itself in a reaction that we propose to be analogous to peptidyl transfer on the ribosome.

The fragment reaction catalysed by the ribosome (Fig. 1, left) results in transfer of formylmethionine (fMet) from a fragment of fMet-tRNA onto either the amino group of puromycin or the hydroxyl group of hydroxypuromycin<sup>2</sup>. We used an analogous reaction (Fig. 1, right) as the basis of an *in vitro* selection for acyl-transferase ribozymes. A pool of approximately 10<sup>15</sup> different RNA molecules was generated by *in vitro* transcription of a synthetic DNA template. Each RNA sequence contained a 90-nucleotide random region flanked by constant regions. Pool RNA was treated with calf intestinal phosphatase to remove the 5'-triphosphate and expose the 5'-hydroxyl group, the desired amino-acid acceptor. The substrate 5'-pCAACCA-O3'-(N-biotinyl-Met) mimics a 3'-fragment of charged tRNA, and was used as the amino-acid donor in the selection. After incubation of pool RNA with substrate, RNA sequences that were able to catalyse transfer of the N-biotinylated methionine to their own 5'-hydroxyl (or any other nucleophile on the RNA) were separated from inactive sequences on streptavidin agarose, by their biotin tag (Fig. 2a). The enriched RNA was converted to cDNA, amplified by the polymerase chain reaction (PCR) and transcribed to generate input RNA for the next round of selection.

Pool RNA was assayed for acyl transferase activity after each round of selection (Fig. 2b). Under selection conditions, the initial random pool showed about 0.03% self-biotinylation per hour. Successful enrichment of active acceptor sequences was readily apparent after four rounds of selection. The selection pressure was then increased by decreasing the incubation time from 30 min to 3 min. In addition, mutagenic PCR<sup>3,4</sup> was performed before rounds 6 and 7 to allow for the evolution of advantageous base changes. After 11 cycles of selection, 20% of the pool RNA self-biotinylated after an incubation of 3 min, a 10<sup>4</sup> increase in acyl transferase activity over pool 0. Non-phosphatased pool 11 RNA (that is, RNA retaining its 5'-triphosphate) showed no activity, suggesting that transfer of the biotinylated methionine to the 5'-hydroxy group was the predominant selected reaction.

RNA molecules from pool 11 were cloned and sequenced. The 25 sequenced clones fell into one dominant class of 22 closely related sequences (class 1), a second small class consisting of two members and one unique sequence. Comparison of the class 1 sequences (Fig. 3A) revealed 90% identity, indicating that these sequences had diverged from a common ancestor. The most striking feature of the class 1 sequence is a 13-nucleotide invariant region close to the 3' end of the originally random region. The 6 nucleotides at the 3' end of this site are complementary to the sequence of the aminoacylated oligonucleotide substrate, whereas

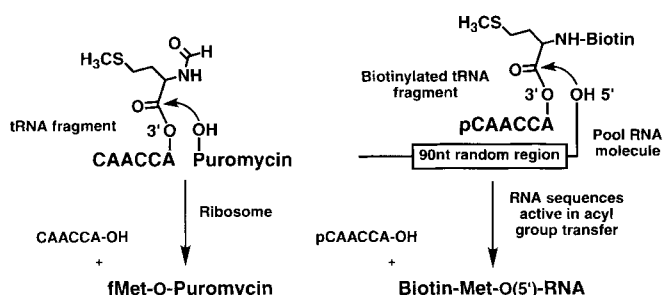


FIG. 1 Chemical analogy between a variant form of the ribosomal 'fragment reaction' (left), and the acyl-transfer reaction used for *in vitro* selection of new ribozymes (right). The *in vitro* selection was based on transfer of a biotinylated methionine from an oligonucleotide substrate to a pool RNA molecule (the synthesis and characterization of this substrate will be described elsewhere). An RNA fragment carrying an N-acylated methionine functions as acyl donor in both reactions.

the 7 nucleotides at the 5' end are complementary to the (constant) 5' end of the ribozyme, except for the two 5'-terminal Gs of the ribozyme which could pair as G:U wobble base pairs (Fig. 3B, c). We hypothesize that this 13-nucleotide sequence acts as an internal template that brings together the aminoacyl group of the substrate and the 5'-hydroxyl at the 5' end of the pool RNA.

All class I clones showed similar acyl-transferase activity. Kinetic analysis of the most active class I sequence (clone 18) showed that it obeys standard Michaelis-Menten-like kinetics for a single turnover reaction with a single saturable binding site, with  $k_{cat} = 0.19 \pm 0.013 \text{ min}^{-1}$ ,  $K_m = 54 \pm 15 \text{ nM}$ , and  $k_{cat}/K_m = 3.8 \times 10^6 \text{ min}^{-1} \text{ M}^{-1}$ . The yield of 5'-acylated ribozyme reached a plateau at 50% after 1 h, suggesting that only about half was correctly folded; kinetic parameters were corrected to reflect 50% functional sequences. Mutants of this clone in which the putative G:U base pairs between the 5' Gs of the RNA and the internal template region were replaced by G:C base pairs (U83C and U84C; Fig. 3B, d) showed a 14-fold lower  $k_{cat}$  than the wild type (Fig. 3B, c). We suggest that the wobble base pairs may unwind the double helix locally and make the reactive centre more accessible for interaction with the functional groups of the ribozyme; alternatively, the exocyclic amino groups of the two Gs may be more available to interact with the ribozyme in G:U wobble pairs than in G:C base pairs, and such interactions may be necessary for the ribozyme to bind and position the 5'-terminal guanosine effectively for catalysis. In contrast, the rate of the templated, but otherwise uncatalysed, acyl transferase reaction was sixfold faster in the context of a completely Watson-Crick duplex (Fig. 3B, b) than for the analogous wobble configuration (Fig. 3B, a). Correctly folded ribozyme (with a G:U template) confers a rate acceleration of 1,000-fold over the G:U templated reaction, and 160-fold over the all Watson-Crick templated reaction. Deletion of all sequences downstream of the putative substrate-binding site resulted in only a minor decrease in acyl transferase activity (Fig. 3B, e). Deletion of the sequence complementary to the substrate abolished activity (Fig. 3B, f).

Because the ribosomal peptidyl transferase activity can synthesize ester linkages as well as the standard amide linkages, we investigated whether our selected acyl transferase could also synthesize amide linkages. We therefore made a derivative of acyl transferase 18 in which the 5'-hydroxyl was replaced with a 5'-amino group. This was accomplished by performing the *in vitro* transcription of the acyl transferase RNA in the presence of 5'-amino, 5'-deoxyguanosine; under optimal conditions, about 70% of the resulting transcripts begin with a 5'-amino group, whereas the remainder begin with a 5'-triphosphate and do not participate in the reaction. This modified ribozyme can transfer the biotinylated methionine from the substrate oligonucleotide to its 5'-amino group almost as efficiently as the normal ribozyme carries out transfer to its 5'-hydroxyl group (Fig. 4A). The catalytic

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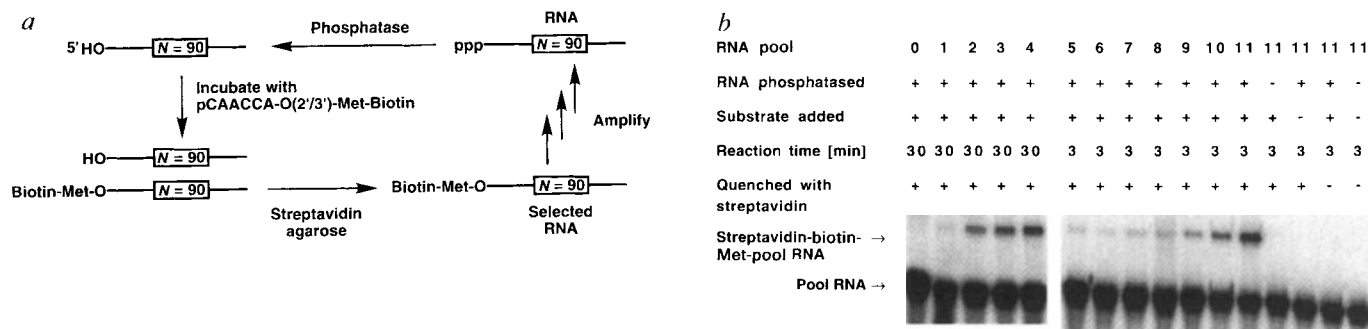


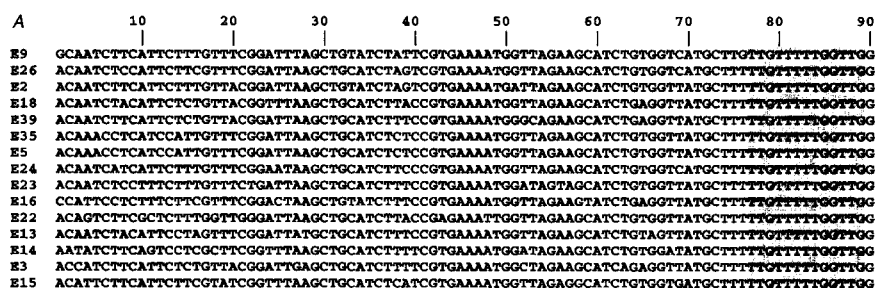
FIG. 2 Selection and evolution of RNA acyl transferases. **a**, A pool of random RNA sequences was incubated with the aminoacylated RNA substrate. RNA sequences that became tagged with biotinyl-methionine were purified on streptavidin agarose, amplified and dephosphorylated to yield a new pool, enriched for acyl transferase ribozymes. **b**, Autoradiogram showing self-acylation activity as a function of selection cycle. Band-shift experiments with streptavidin allowed separation of biotinylated acceptor sequences from unmodified pool RNA.

**METHODS.** The RNA pool (random region, 90 nucleotides (nt); complexity,  $10^{15}$ ) was synthesized as previously described<sup>3</sup> with constant regions 5'-GGAACAACUUGCAGCUCUGA-3' (5' end), and 5'-GUCGUACAUCGUAAGUC-3' (3' end). Four equivalents of pool RNA were dissolved in reaction buffer (500 mM KCl, 25 mM HEPES, pH 7.3) at 1  $\mu$ M, heated at 80 °C for

5 min, cooled to 20 °C over 10 min and  $MgCl_2$  added to 50 mM. After 5 min, substrate (10  $\mu$ M) was added and the mixture was incubated at 20 °C for 30 min (3 min in later rounds). The reaction was stopped by precipitating the RNA with cold ethanol. The RNA pellet was dissolved in 1 M NaCl, 5 mM EDTA, 50 mM HEPES, pH 7.3 and incubated with 4 ml streptavidin agarose (Pierce) for 30 min at room temperature. Unbound sequences were eluted with 40 column volumes each of high-salt buffer, water, 4 M urea followed by another wash with water. RNAs bound by streptavidin agarose were eluted by heating in the presence of 10 mM biotin at pH 7 (ref. 8), amplified and dephosphorylated to generate a new RNA pool. Round 2 and later rounds were started with 10-fold less RNA, and only 0.4 ml streptavidin agarose was used to bind biotinylated sequences. Band-shift experiments with streptavidin were as described for DNA<sup>9</sup>.

FIG. 3 The class 1 acyl transferases. **A**, Sequence alignment of the random region. The shaded region is 100% conserved. The first 7 nucleotides (nts) of this region are complementary to the constant 5' end of the pool RNA and the following 6 nts are complementary to the substrate. **B**, Templated and ribozyme-catalysed reactions. First-order rate constants were determined under substrate-saturating conditions.

**METHODS.** PCR DNA from round 11 was cloned into pT7 Blue T-Vector (Novagen) and sequenced using the dideoxy method. All clones were transcribed and the RNA phosphatased and assayed for acyl transferase activity. Acceptor RNA 7-mer was obtained by T7 transcription; RNA 13-mer templates were chemically synthesized and HPLC purified by high-performance liquid chromatography. Derivatives of acyl transferase 18 were made by PCR amplification with altered 3'-primers. RNAs (20  $\mu$ M substrate and 20  $\mu$ M template in **a** and **b**, 1  $\mu$ M radiolabelled RNA clone 18 for **c** and **d**) were heated in reaction buffer to 70 °C for 3 min, and annealed at 20 °C for 10 min.  $MgCl_2$  was added to 50 mM; after 5 min at 20 °C, acceptor oligonucleotide was then added in **a** and **b** (1  $\mu$ M) and substrate (5  $\mu$ M) was added in reactions **c**–**f**. Portions of the reaction solution were quenched in 8 M urea, 70 mM EDTA, Tris-Cl, pH 7.0; 5'-acylated acceptor and unmodified RNA were separated on a 20% polyacrylamide gel (**a** and **b**) or by streptavidin band shift (**c**–**f**) and quantified using a phosphorimager (Molecular Dynamics). Initial rates for clone 18 were determined at 6 different substrate concentrations, ranging from 10 nM to 1 mM.  $K_m$  and  $k_{cat}$  were obtained from a nonlinear least-squares fit to the data.



| B                                 |                                                                                                               | $k_{3' \rightarrow 5'} \text{ (min}^{-1}\text{)}$ |
|-----------------------------------|---------------------------------------------------------------------------------------------------------------|---------------------------------------------------|
| Acyl transfer reactions at pH 7.3 |                                                                                                               |                                                   |
| a                                 | <p>Biotin-Met<br/>3' O OH 5'<br/>pCAACCA <b>GG</b>AACAA<br/>3' GUUGGU <b>UU</b>UUGUU</p>                      | $2.5 \times 10^{-4}$                              |
| b                                 | <p>Biotin-Met<br/>3' O OH 5'<br/>pCAACCA <b>GG</b>AACAA<br/>3' GUUGGU <b>CC</b>UUGUU</p>                      | $1.6 \times 10^{-3}$                              |
| c                                 | <p>Biotin-Met<br/>3' O OH 5'<br/>pCAACCA <b>GG</b>AACAA<br/>3' — GUUGGU <b>UU</b>UUGUU</p> <div>clone18</div> | $2.5 \times 10^{-1}$                              |
| d                                 | <p>Biotin-Met<br/>3' O OH 5'<br/>pCAACCA <b>GG</b>AACAA<br/>3' — GUUGGU <b>CC</b>UUGUU</p> <div>clone18</div> | $1.8 \times 10^{-2}$                              |
| e                                 | <p>Biotin-Met<br/>3' O OH 5'<br/>pCAACCA <b>GG</b>AACAA<br/>3' GUUGGU <b>UU</b>UUGUU</p> <div>clone18</div>   | $9.4 \times 10^{-2}$                              |
| f                                 | <p>Biotin-Met<br/>3' O OH 5'<br/>pCAACCA <b>GG</b>AACAA<br/>3' <b>UU</b>UUGUU</p> <div>clone18</div>          | no acyl transfer<br>observed                      |

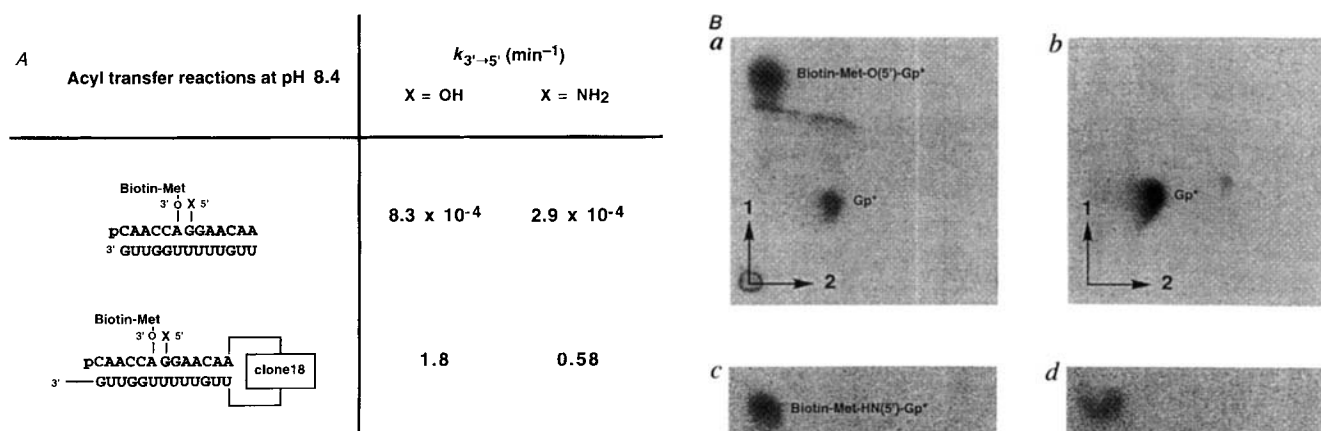


FIG. 4 Ester and amide bond formation by acyl transferase 18. A, First-order rate constants for acyl transfer to the 5'-hydroxy or to the 5'-amino group in the templated and the ribozyme-mediated reaction. B, Alkaline stability/lability of products of acyl transfer to 5'-hydroxy and 5'-amino ribozyme. Biotinylated GMP derived from the 5'-hydroxy ribozyme was readily cleavable to 3'-GMP in aqueous hydroxide (TLCs a and b), whereas biotinylated GMP derived from the 5'-amino ribozyme was stable under identical conditions (TLCs c and d).

METHODS. 5'-amino RNA was synthesized by using 5'-amino-5'-deoxyguanosine as the initiating nucleotide for T7 transcription of acyl transferase 18 PCR-DNA, using 4 mM 5'-amino-5'-deoxyguanosine and 0.25 mM GTP. Full-length transcripts (5'-amino versus 5'-triphosphate ratio of 7:3) were purified on a 5% polyacrylamide gel and assayed for acyl transfer activity at pH 8.4 (EPPS buffer). Observed rates were corrected for 50% functional sequences. RNA acyl-transferase 18 uniformly labelled by transcription with [ $\alpha$ -<sup>32</sup>P]GTP was incubated with substrate followed by digestion with ribonu-

lease T2 at pH 4.4. The products were incubated with streptavidin agarose and unbound nucleotides were washed away. Nucleotides bound to streptavidin were eluted and resolved by two-dimensional TLC on cellulose plates<sup>10</sup>. Hydrolysis was performed in 0.5 M sodium hydroxide at room temperature for 2 h. The position of guanosine-3'-monophosphate was confirmed by ultraviolet shadowing after development of the TLC with an authentic sample (Sigma). The minor spots in TLCs a, c and d are probably due to oxidized forms of biotin-Met-O(5')-Gp.

rate constant determined for the ribozyme catalysed amide-bond formation ( $0.58 \text{ min}^{-1}$ ; Fig. 4A) compares well with the first-order rate constant of  $8 \text{ min}^{-1}$  determined for the peptidyl transferase in the fragment reaction<sup>5</sup>. That no acyl transfer was observed if the catalytic RNA carried a 5'-triphosphate or a 5'-monophosphate suggested that the 5'-hydroxyl or 5'-amino groups of the 5'-terminal guanosine nucleotide were the nucleophilic acceptors for the aminoacyl group, as expected from the template-induced proximity of the 5'-group of the ribozyme to the aminoacyl group of the substrate<sup>6</sup> (A. T. Profy and D. A. Usher, personal communication). To provide further evidence that the expected 5'-ester and 5'-amide had been formed, aminoacylated ribozyme RNA was digested with mononucleotides with ribonuclease T2, and the biotinylated nucleotides were purified away from the non-biotinylated digestion products on streptavidin agarose and resolved by two-dimensional thin-layer chromatography (2D-TLC) (Fig. 4B). The products derived from 5'-hydroxy and 5'-amino RNA had identical mobilities but were chemically distinct in that the product derived from 5'-OH RNA was sensitive to alkaline hydrolysis but the product derived from 5'-amino RNA was stable under identical conditions. The simplest explanation of the fact that distinct products were obtained when

the 5'-substituent of the RNA was changed is that the 5'-substituent is the acyl acceptor, and that the products are the expected base-labile 5'-ester and base-stable 5'-amide.

We interpret our results to show that RNA alone can accelerate the chemical reaction at the heart of protein synthesis, as has been previously suggested by the finding that largely deproteinized ribosomes still retain peptidyl transferase activity<sup>7</sup>. The isolated ribozyme appears to be able to synthesize both ester and amide linkages, as does the ribosomal peptidyl transferase<sup>2</sup>. The parallels between the isolated ribozyme and ribosomal peptidyl transferase suggest that the evolutionary precursor of rRNA might have been a ribozyme with 3'-OH  $\rightarrow$  3'-OH acyl-transferase activity, for example one that transferred an acyl group from a self-acylating RNA to another RNA. Such a ribozyme might easily evolve into one that could transfer an aminoacyl group to the amino group of a 3'-aminoacylated RNA, perhaps leading to the synthesis of random peptides. The ability to synthesize specific peptide products could then evolve by the acquisition of specificity for the aminoacyl-RNA donors, as long as these were amino-acid specific. Recognition of the RNA component of the aminoacyl-RNA donors by the formation of base pairs would provide a natural pathway for the evolution of coded protein synthesis. □

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CORRESPONDENCE and requests for materials should be addressed to J.W.S. (e-mail: szostak@molbio.harvard.mgh.edu).



# Simplified hot start PCR

David E. Birch et al.\*

**The use of a thermally activated DNA polymerase PCR gives improved specificity, sensitivity and product yield without additives or extra process steps.**

HIGH background and low specific product yield can occur in a polymerase chain reaction (PCR) when reaction components are mixed at room temperature<sup>1-3</sup>. Reaction setup below the optimal primer annealing temperature (usually 50–65 °C) permits nonspecific primer annealing and extension. Undesired, non-specific primer extension products formed this way may be amplified in the PCR, resulting in misprimed products and primer oligomers.

In 'hot start' PCR, at least one essential reagent is withheld from the PCR mixture until the system has reached a temperature that favours specific primer annealing. Hot start PCR has been implemented by mechanically separating a reagent<sup>1-3</sup> or by blocking the polymerase with an antibody<sup>4,5</sup>. In another variation, nonspecific product formed at low temperatures was destroyed by a heat-labile uracil glycosylase<sup>6</sup>. Hot start can greatly improve specificity, sensitivity, and yield in a PCR<sup>1-6</sup>.

AmpliTaQ Gold is a modified, inactive form of AmpliTaq DNA polymerase (both from Perkin-Elmer, Foster City, California). AmpliTaq is a thermostable DNA polymerase commonly used for PCR. Though the maximum activity of most polymerases used for PCR is at

60–70 °C, these enzymes exhibit significant activity at room temperature, whereas AmpliTaq Gold is inactive at room temperature. Heat restores the polymerase activity, allowing the addition of a hot start to an existing PCR, with no changes to the protocol except the addition of a pre-PCR heat cycle. PCR setup can be done at room temperature, as primer extension cannot occur when the DNA polymerase is inactive. The pre-PCR heat cycle can be programmed into the thermal cycler so that the transition from enzyme activation to PCR cycling occurs without going below the optimal primer annealing temperature.

## Modified enzyme features

For a thermostable DNA polymerase provided in an inactive state, the enzyme unit activity given is the nascent DNA polymerase activity. To measure the nascent activity, enzyme samples are heated for 3 h at 80 °C in a buffer containing 25 mM Tris-HCl, pH 8.0 (at room temperature), 50 mM KCl, 1 mM  $\beta$ -mercaptoethanol, 0.5 per cent NP-40 and 0.1 per cent gelatin, and are then assayed in a DNA polymerase activity assay. Under these conditions the

enzyme is activated maximally without thermally attenuating the final polymerase activity (data not shown).

To demonstrate the activation during a pre-PCR heat cycle, the DNA polymerase was incubated at 95 °C, in a commonly used PCR buffer (Fig. 1). The enzyme was activated maximally within 15 minutes. It is expected, however, that some of the potential polymerase activity was lost due to the extended incubation at 95 °C.

The new modified enzyme was substituted for unmodified DNA polymerase in a PCR amplification of a 142 bp HIV target from ten template copies in a background of 10 ng of human placental DNA (Fig. 2). This system was previously used for PCR

without a hot start, using oligomer hybridization with <sup>32</sup>P-labelled probes for product detection<sup>7</sup>. For manual hot start, unmodified DNA polymerase was withheld from the reaction mixture, then individually added to each PCR tube during a 78 °C hold before the PCR cycling began. For modified polymerase reactions, all of the reagents were mixed at room temperature, and the only change to the PCR conditions was the addition of a nine minute, 95 °C pre-PCR heat cycle. The product was analysed by agarose gel electrophoresis with ethidium bromide staining. In the reactions done without hot start, specific product was not visible (Fig. 2, lanes 4 and 5). Manual hot start with unmodified DNA polymerase gave visible product (Fig. 2, lanes 6 and 7). However, PCR with the modified DNA polymerase

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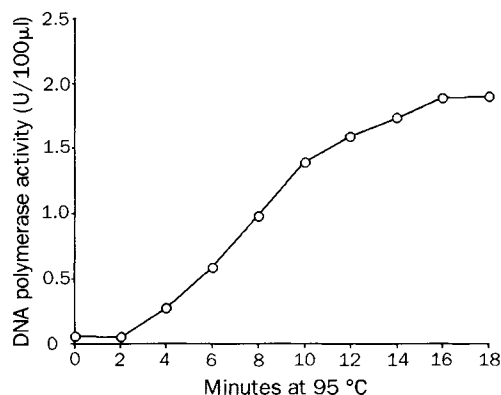


FIG. 1 Heat activation of AmpliTaq Gold. To simulate PCR conditions, AmpliTaq Gold was added at a concentration of 2.5 Units per 100  $\mu$ L to a buffer containing 10 mM Tris-HCl, pH 8.3 (at room temperature), 50 mM KCl, 2.5 mM MgCl<sub>2</sub> and 200  $\mu$ M each of dATP, dCTP, dGTP, and dTTP. Aliquots of 100  $\mu$ L were added to PCR tubes and covered with 2 drops of mineral oil. The tubes were closed and put into a Perkin-Elmer GeneAmp DNA Thermal Cycler 480 on hold at 25 °C. The cycler was programmed to ramp to 95 °C. Two tubes were withdrawn and put on ice before starting the program, then every two minutes after reaching the target temperature. The samples were assayed for polymerase activity, as described in ref. 10.

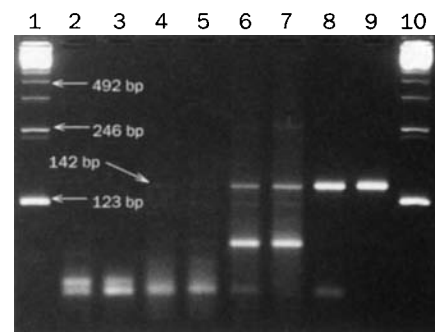


FIG. 2 100  $\mu$ L PCR amplifications of a 142bp product of the HIV-1 genome (Perkin-Elmer) were run in duplicate using manual or no manual hot start methods. This procedure used 2.5 Units of AmpliTaq DNA Polymerase or AmpliTaq Gold, with 1  $\times$  PCR Buffer II, 2.5 mM MgCl<sub>2</sub>, 200  $\mu$ M each of dATP, dCTP, dGTP, 400  $\mu$ M dUTP, 50 pmoles of primers SK145 and SK431 (ref. 7), 1 unit of UNG, and 10 input copies of HIV-1 Positive Control DNA in 10 ng of HIV-1 Negative Control DNA in MicroAmp reaction tubes and a GeneAmp PCR System 9600. Following 43 cycles of amplification (1 initial preincubation cycle at 95 °C for 9 min; plus 43 cycles at 95 °C for 30 sec and 60 °C for 1 min, plus a final cycle at 60 °C for 10 min), the samples were analysed on an ethidium bromide stained 3 per cent Nusieve GTG and 1 per cent SeaKem GTG agarose gel (FMC BioProducts, Rockland, Maine) for 2 hours per 25 cm at 200 volts. Lanes 2 to 3: 0 input copies, AmpliTaq DNA polymerase, no preincubation, no manual hot start; lanes 4 to 5: 10 input copies, AmpliTaq DNA polymerase, no preincubation, no manual hot start; Lanes 6 to 7: 10 input copies, AmpliTaq DNA polymerase, no preincubation, manual hot start; Lanes 8 to 9: 10 input copies, AmpliTaq Gold, 9 min preincubation, no manual hot start; Lanes 1 and 10: 123bp DNA ladder, 1  $\mu$ g.

gave a stronger product band and reduced nonspecific products (Fig. 2, lanes 8 and 9). The increased specific product yield with AmpliTaq Gold is consistent with increased functional enzyme activity ('timed release') in later cycles, when enzyme activity usually becomes limiting.

## Applications

The increased amplification specificity of modified DNA polymerase is illustrated by improvements seen in the co-amplification of the genes encoding the small subunit ribosomal RNA (16S rRNA) and the  $\beta$ -subunit of RNA polymerase (*rpoB*) from *Mycobacterium tuberculosis*, the causative agent of tuberculosis (TB). Amplification of the 16S rRNA gene allows the detection and identification of the pathogen<sup>8</sup>. Mutations in the *rpoB* gene have been linked to resistance to rifampin<sup>9</sup>, one of the primary drugs used in the treatment of TB. Co-amplification of both target genes is desirable because the presence *M. tuberculosis* and its rifampin resistance/sensitivity genotype can be determined from products of a single amplification reaction. The relative performance of the unmodified and the modified DNA polymerase on the co-amplification assay was examined. DNA isolated from *M. tuberculosis* was amplified with unmodified or modified polymerase in a bicine-buffered reaction in a thermal cycler. As shown in Fig. 3, significant amounts of nonspecific amplification products were formed when co-amplification was carried out with unmodified DNA polymerase, especially in the presence of heterologous DNA. In contrast, when amplification was carried out with the modified enzyme, nonspecific products were greatly reduced and the amplification efficiency of the specific targets improved. This was apparent in the *rpoB* product, which showed a barely visible band in the unmodified-

enzyme amplification, but was a more prominent band in the modified-enzyme amplification. Furthermore, the yields of the two products were much more balanced with the modified enzyme.

The ability to amplify multiple targets in a single PCR has great utility. Examples include the identification of several pathogens in a biological sample, mutational analysis of multiple gene exons, and simultaneous interrogation of multiple loci for human genetic linkage or oncogenic studies. Benefits of multiplexing include experimental simplification, decreased effort, greater cost effectiveness and reduced time. However, increasing the number of primer pairs in a PCR exacerbates the poor specificity and low product yield that result from primer interactions and mis-priming. Extensive primer testing and reaction optimization are required to minimize these problems when using an unmodified DNA polymerase. With a modified enzyme amplifications can be done simultaneously with little or no primer sequence optimization. A comparison of multiplex amplifications using unmodified DNA polymerase and the modified form is shown in Fig. 4. The reactions with the modified enzyme gave reduced primer artefact and background, leading to increased yield and specificity relative to the unmodified DNA polymerase reactions. In addition, one of the PCR products (100 bp) was not amplified with unmodified polymerase, but was clearly detected in the modified polymerase reactions. The polymorphic markers shown in Fig. 4 have been co-amplified with 20 exons from the cystic fibrosis gene using the modified enzyme to give a total of 34 products clearly resolved by agarose gel (data not shown, G. A. Zangenberg).

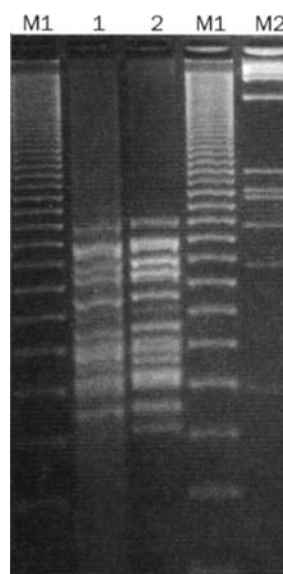
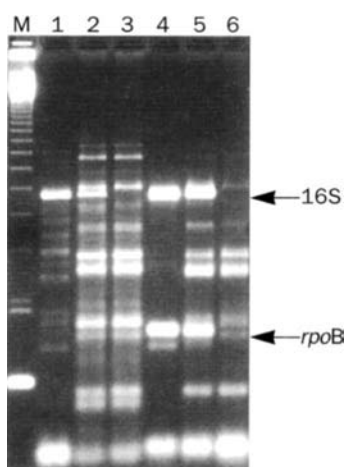


FIG. 4 Amplicon generated at 18 different DNA loci using 2 ng of genomic DNA, separated on a 3.5 per cent MetaPhor (FMC BioProducts) agarose gel. M1 = Superladder-low, 20 bp ladder (GenSura, Del Mar, California), M2 = (X 174 DNA-Hae III Digest (New England Biolabs, Beverly, Massachusetts)). Lane 1 shows multiplex PCR results using AmpliTaq DNA polymerase, lane 2 shows the same reaction but using AmpliTaq Gold with dramatically decreased primer-dimer (size range of 40 to 60bp). PCR product sizes range from 100 bp to 242 bp.

FIG. 3 PCR products from co-amplification of 16S and *rpoB* gene targets from ten copies of *Mycobacterium tuberculosis* DNA. Lanes 1-3: products generated with AmpliTaq DNA polymerase (Perkin-Elmer). Lanes 4-6: products generated with AmpliTaq Gold (Perkin-Elmer). Target DNA was amplified alone (lanes 1 and 4), and in the presence of DNA from  $10^5$  human leukocytes (lanes 2 and 5). Products from the amplification of leukocyte DNA alone are in lanes 3 and 6. Lane M: 1  $\mu$ g of 123 base pair DNA marker (Gibco BRL, Gaithersburg, Maryland).



Aliquots of 10  $\mu$ l volume were electrophoresed through two per cent NuSieve GTG and one per cent SeaKem Agarose (both, FMC BioProducts) in Tris-borate/EDTA buffer, and stained with ethidium bromide. Locations of the specific amplification products are denoted by the arrows.

## Extending the technology

AmpliTaq Gold can be substituted directly for conventional thermostable DNA polymerases in most PCR systems using a standard PCR buffer (GeneAmp 10 $\times$  PCR buffer or 10 $\times$  PCR buffer II and  $MgCl_2$  solution, Perkin-Elmer), when a pre-PCR heat cycle is

used. However, optimizing the reaction conditions, such as pH, KCl concentration or  $MgCl_2$  concentration, may further improve PCR results. Because of the enhanced specificity of a modified DNA polymerase PCR, increasing both the enzyme concentration and cycle number may increase specific product yield without increasing background. The pre-PCR heat cycle can be eliminated, allowing the enzyme to activate during cycling. If a pre-PCR heat cycle is not used, ten or more extra cycles may be necessary to give equivalent product yield (data not shown). The simplicity of the activation makes the enzyme well suited for applications like automated PCR and *in situ* PCR, as well as in high-throughput situations where pre-mixing and aliquotting of reagents is required.

D. E. Birch, L. Kolmodin, J. Wong, G. A. Zangenberg and M. A. Zoccoli are with the Department of Product Development; N. McKinney and K. K. Y. Young are with the Department of Infectious Diseases, Roche Molecular Systems, Alameda, California 94501, USA. W. J. Laird is an independent consultant. We thank D. Gelfand, J. Sninsky, J. Raymond (Roche Molecular Systems) and J. Horak (Perkin-Elmer, Foster City, California 94404 USA) and K. Jeong and S. Kurita.

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# Molecules and modalities

Product review has the goods and wares for studying the molecular mechanisms of the cell — items include an automated membrane developing system, a mutation detection system and high-efficiency cloning kits.

PE APPLIED BIOSYSTEMS now offers **custom DNA services** in Europe. This facility will supply oligonucleotides to both the UK market and countries throughout the European Community. One of the main restrictions on projects, such as the human genome mapping and sequencing initiative, is the cost of the primers used both in the PCR and for DNA sequencing. By utilizing new high-throughput technology the company should be able to significantly reduce the cost of making and supplying oligos particularly fluorescent primers and TaqMan probes used with the ABI Prism range of automated systems. The ReadyPure oligos supplied by the custom DNA facility are purified by reversed-phase chromatography and are provided at no extra charge.

**Reader Enquiry No. 100**

Clontech now offers its **two-hybrid, cDNA library construction kit** for constructing libraries in the GAL4 activation domain vector pGAD10. Resulting libraries can be screened using the Matchmaker two-hybrid system (or other GAL4-based or suitable LexA-based two-hybrid systems). The two-hybrid system is a yeast-based genetic assay used to detect protein-protein interactions *in vivo* by functional restoration of the yeast transcriptional activator GAL4. The kit can also be used for screening DNA-protein interactions using the Matchmaker one-hybrid system. The two-hybrid kit comes complete with sufficient reagents to construct three cDNA libraries from 10 µl of the mRNA of choice. The kit includes linearized, dephosphorylated pGAD10 vector, a positive control mRNA, complete library construction and library screening protocols, as well as two yeast reporter strains (SFY526 and HF7c), which can be used for detecting positive interactions via a β-galactosidase assay or histidine growth selection. Also available is a range of pre-made activation-domain cDNA and genomic libraries from a wide variety of tissues and species. These are provided in a variety of activation-domain vectors, such as pGAD, pGADGH, pGADGL, λACT and pACT2.

**Reader Enquiry No. 101**

## PCR products

Stratagene's pCR-Script SK(+) **cloning kits** are said to be an efficient way to clone PCR products, regardless of the enzyme used to perform the PCR. A

newly optimized cloning procedure allows PCR products to be quickly and easily cloned, with only a one-hour ligation. According to the manufacturer, the protocol for performing PCR, ligating to the pCR-Script vector, and transforming and plating the bacteria can be accomplished easily in one afternoon. In addition, no extra nucleotides are needed on PCR primers. Recent improvements give the pCR-Script cloning kits consistently higher cloning efficiencies than T/A methods when using either *Taq* polymerase or proof-reading enzymes, such as *Pfu* DNA polymerase. When cloning the 1.1-kb test insert, quality control for each kit requires at least 80 per cent white colonies, with ≥90 per cent of the recombinants containing an insert. The cloning kits provide all the reagents necessary to perform PCR, including PCR of recombinant colonies.

**Reader Enquiry No. 102**

FMC BioProducts has available a new brochure outlining the wide range of high-performance products specifically designed for **PCR research**. The company has developed a brochure that describes its products for PCR product separation and analysis. The catalogue covers products for a wide variety of techniques, such as separating micro-satellite repeat sequences that differ by as little as 2 bp or recovering large PCR products from gels.

**Reader Enquiry No. 103**

MJ Research has developed the PTC-225-Tetrad **thermal cycler**, a four-headed thermal cycler for high-throughput cycle sequencing and diagnostic screening programmes. With true network capabilities, several thermal cyclers can be linked together or linked to other laboratory equipment through a desktop computer. A desktop computer can also be used to control all functions of the thermal cycler, including temperature and speed. A robotic control sequence communicates with laboratory robots, allowing fully automated sequencing. The four individual alpha blocks sit on a single control base facing inwards and with heated lids that face outwards.



**MJ Research's PTC-225-Tetrad thermal cycler.**

Optional mechanical lids automatically open outwards at the end of the programme, to allow robotic access. Each thermal cycler can accept four single or double alpha blocks and these can be individually controlled from the programmable control unit. In addition, they can accept tubes, plates or microslides at the same time.

**Reader Enquiry No. 104**

The new LigA-Tor kits from R&D Systems are designed to allow rapid, high-efficiency **cloning of fragments generated by PCR**. Non-proof-reading DNA polymerases, such as *Taq*, often add single A overhangs to the 3' ends of amplified products. The kits are designed to take advantage of this process. The pTAG vector provided with the kits is pre-cut and has complementary T-overhangs for direct ligation to the PCR fragment. The pTAG vector has a carefully designed polylinker for easy subcloning of the captured PCR fragment into a wide range of vectors. It also contains two different selectable markers to help eliminate false-positives that may arise because of template carry over. The competent cells in the kits are subaliquoted into two reaction sizes to avoid excessive freeze-thaw cycles and to maintain a high transformation efficiency. This direct method of PCR cloning is said to eliminate the need for restriction digestions, linkers, adaptors or special primers for PCR. A visual blue/white screen allows easy identification of positive clones. Different kits are available for experienced users and researchers new to the technique. Complete sets of reagents, critical reagents only or pTAG vector alone can be purchased (10- and 40-reaction kits are available).

**Reader Enquiry No. 105**



# Synthesis and sequencing

Cambio has introduced Epicentre Technologies' IsoTherm DNA polymerase, a sequencing tool that is said to offer enhanced performance of isothermal DNA sequencing of difficult templates. Having an optimal activity at 65 °C, this DNA polymerase is effective on regions of secondary structure or sequences with high G + C content where lower-temperature isothermal enzymes can fail. According to the manufacturer, reaction times are shorter, using less enzyme and template, thus giving faster results at lower cost. Additionally, the DNA polymerase is thermostable, remaining active even after two weeks at 25 °C, the company states. The sequencing protocols are easy to perform and can be used with a variety of labelling methods in manual sequencing procedures. Separate kits are available for internal and end-labelling.

Reader Enquiry No. 106

Beckman Instruments has announced a new version of the UltraFast DNA synthesis chemistries, for use with ABI synthesizers from Perkin-Elmer. These reagents are designed to reduce significantly the time needed for post-synthesis cleavage

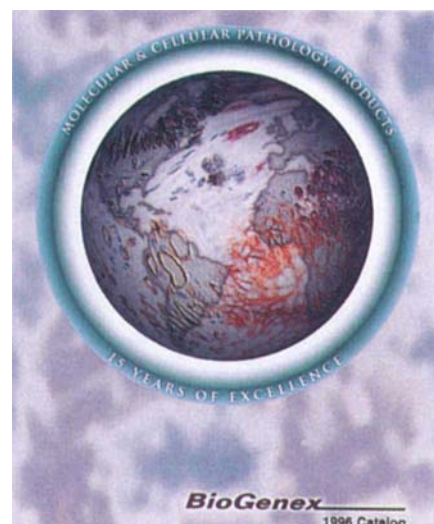
and deprotection. Users should be able to reduce the time for on-line cleavage, and for off-line deprotection, from 2.5 h to about 10 min. The reduction in cleavage and deprotection processing time is achieved by adding methylamine to the ammonia cleavage and deprotection reagent and an acetyl group to protect the exocyclic amine of the dC-phosphoramidite, instead of the usual benzoyl-protecting group. β-Cyanoethyl phosphoramidites are packaged in 7-, 10- and 60-ml bottles in quantities ranging from 0.25 g to 5.0 g. For bulk users, 10-g phosphoramidites are packaged in 30-ml bottles. Larger quantities can be ordered in multiples of 100-g units. Two sizes of β-cyanoethyl deoxyinosine phosphoramidite are also available, as well as base diluent, activator, non-coupling reagents, and reactor columns.

Reader Enquiry No. 107

Life Technologies, producer of Gibco BRL products, now offers the Powerscan system, which utilizes Cleavase fragment-length polymorphism (CFLP) for mutation detection. The CFLP system is designed to detect polymorphisms and mutations in PCR products between 0.1 and 2.0 kb in length. It is said to allow users to screen larger amplicons (up to ten times greater) than with conventional methods, which means that there is more sequence scanned per reaction for increased throughput. The system may prove to be as sensitive as sequencing (> 98 per cent), but for a fraction of the cost. The cornerstone of this system is the Cleavase I enzyme, a thermostable enzyme that contains structure-specific, single-strand endonuclease activity. 'Mutant' molecules are distinguished from 'wild-type' molecules by the increase or decrease in the signal intensity of one or more bands. Direct sequencing of the regions where these changes occur correlate well (within 40 bp) with actual mutation sites. The size distinguishing bands can be used to identify the position of mutations relative to the labelled end. Cleavase I enzyme products can be detected by radioactive or non-radioactive methods.

Reader Enquiry No. 108

The 1996 BioGenex molecular and cellular pathology products catalogue is now available, featuring the OptiMax automated immunostainer and listing more than 250 monoclonal and polyclonal antibodies, as well as DNA-probe test kits and enhanced biotin-streptavidin detection systems for use in immunohistochemistry and *in situ* hybridization. This year's catalogue lists many new primary antibodies, including monoclonals to breast tumour marker (CA-15-3), CD3, CD35, CD45RB, CD54, CD66, *c-erbB-3* protein, *c-myc* protein, ovarian tumour marker (CA-125) and



The 1996 BioGenex products catalogue.

placental alkaline phosphatase. A new product for use with the popular microwave antigen retrieval method is the high-pH, antigen-retrieval AR-10 solution. Formulated for use with automated immunostainers, the new haematoxylin counterstain for automated systems is said to offer improved development time. Of particular interest may be the new features for the OptiMax immunostainer including bar-code labelling, increased reagent capacity and automatic cleaning cycles.

Reader Enquiry No. 109

## DNA measurement

Spectronic Instruments has released its new biochemistry SoftCard for DNA quantification using its for the Genesys 2 and Genesys 5 spectrophotometers. The Softcard contains various programs that should prove useful for molecular biology research, including DNA measurement capabilities, which can be used to measure simultaneously DNA ratio, and DNA, RNA and protein concentration. For protein measurements, there is program that can measure the concentration of proteins using BCA, Biuret, Bradford or Lowry standard curves. There is the capability for direct ultraviolet measurement that can measure the concentration of proteins, dsDNA, ssDNA or ssRNA, as well as oligonucleotides from direct ultraviolet measurements. Also provided is column monitoring, to monitor and plot the absorbance (or concentration) of fractions collected from a liquid chromatography column. All programs on the biochemistry SoftCard will run in English, French, German, or Spanish.

Reader Enquiry No. 110

Incstar has introduced new DNA research technology developed by Sorin, the Gen-eti-k DNA enzyme immunoassay (DEIA). The manufacturer states that this assay technology can be used to detect

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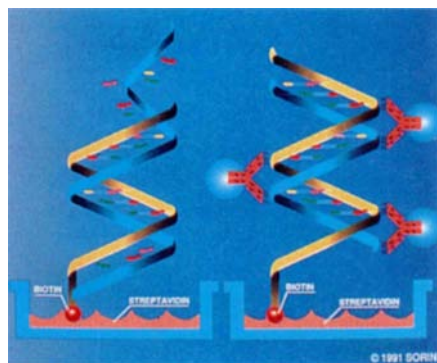
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READER ENQUIRY NO 334

specific DNA sequences in a wide range of concentrations and molecular sizes. It is may be particularly suited to the detection of non-amplified DNA or DNA amplified by any documented amplification technique. This immunoassay uses non-isotopic methodology to detect double-stranded DNA formed by the hybridization of target DNA with specific genetic material. It is a microtitre-well enzyme immunoassay method, which is fully compatible with standard equipment for pipetting, washing and reading results. It should facilitate the processing of a large number of samples in research laboratories. In less than four hours, the assay detects the hybridization event, independent of the nucleic acid sequences involved in the formation of the specific hybrids. The DNA assay has two original features. The general assay scheme and the reagents used are the same as those employed in an enzyme immunoassay. The assay's second feature is the antibodies' ability to resolve the problem of labelling the DNA with a signal source. It is the hybridization itself that generates the epitope recognized by the antibody. The probe-DNA hybrid being examined is detected using an anti-DNA monoclonal antibody, which reacts only with



Incstar's DNA enzyme immunoassay.

dsDNA. When the denatured DNA samples are dispensed into the wells, the probe specifically binds the complementary hemistrand, if present, to form a hybrid. After incubation and subsequent clearing of the sample, the addition of the anti-dsDNA antibody identifies the wells where hybridization has taken place and the wells where the probe has not bound the DNA. Finally, the addition of an enzyme tracer is used to detect the DNA-antibody bond.

**Reader Enquiry No. 111**

PanVera has announced the availability of its Beacon **DNA quantification kit**. Designed for ease of use, this kit contains a fluorescent dye that selectively binds to double-stranded DNA. The method allows for specific quantification of double-stranded DNA, with minimal detection of single-stranded nucleic acids.

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The procedure involves generating a standard curve by serial dilution of a DNA standard. The kit can be used to generate both high (3–200 ng) and low (0.098–25 ng) DNA standard curves. According to the manufacturer, the kit produces a coefficient of variation for the standard curve  $>0.999$ , a limit of quantitation (mean  $\pm 10$  s.d.) of  $<3.0$  ng DNA for the high standard curve and  $<0.98$  ng DNA for the low standard curve.

**Reader Enquiry No. 112**

To achieve **DNA detection** down to 0.05 pg of DNA in Southern blotting in under two hours, Amersham offers the dioxetane-based CDP-Star detection reagent for its Gene Images system. Because the initial light output of dioxetane-based reagent is high, complete protocols, from probe labelling to blot detection, can be completed in a single day. The system is available as a complete labelling and detection system, including all of the reagents necessary to perform complete protocols. Two sizes are available for detection of 2,500 cm<sup>2</sup> membranes: one for 30 labelling reactions and the other for 60 labelling reactions. The random prime labelling and the CDP-Star detection modules are available separately. To ensure its performance this product is tested to stated specifications: Southern blot detection of a single copy of genomic DNA (equivalent to 0.05 pg) from a 250 ng loading, and northern blot detection of the p53 message (1.7 kb) from a 1.0 µg loading. It is also recommended for use with Hybond N<sup>+</sup> (for blotting) and Hyperfilm ECL or Hyperfilm MP (for detection).

**Reader Enquiry No. 113**

A new application note has been issued by Hitachi Scientific Instruments, covering the use of the U-2001 ultraviolet/visible spectrophotometer, equipped with an application card for performing **DNA measurements**. The application card is said to provide all the appropriate software for the rapid, accurate and reliable measurement of a variety of parameters for both single- and double-stranded DNA, RNA, oligonucleotides and proteins. The application note reviews both the practical requirements for nanomolar measurements and the calculation routines used, including the Warburg-Christian method for protein concentration. Other measurement parameters include concentration, molar concentration, purity and calculation of melting point for oligonucleotides.

**Reader Enquiry No. 114**

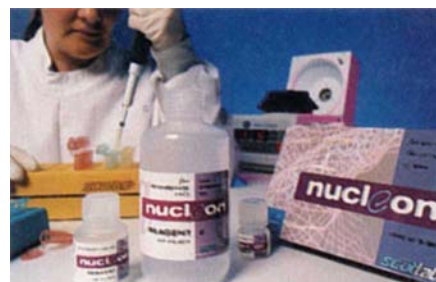
### Plant biology

A complete system for **isolating DNA from plant-leaf tissue** is now available from Boehringer Mannheim. The plant DNA isolation kit is said to offer several advantages over conventional plant DNA

isolation methods. It is designed to eliminate the need for hazardous organic reagents and produces DNA that can be used for PCR, restriction digests or Southern blots. This kit is designed to be flexible, adapting to DNA amounts from 4 mg (a single leaf disk) to 1.0 g of tissue in each sample. Compared to the standard mortar and pestle grinding method, the grinding of spheres requires less time and less effort, making it possible to process multiple samples simultaneously.

**Reader Enquiry No. 115**

ScotLab Bioscience has introduced the Nucleon Phytopure **plant DNA extraction kit** developed with the specific needs of the plant scientist in mind. The kit takes a novel approach by application of silica and borate chemistries to extract DNA free of the polysaccharide contamination



ScotLab's plant DNA extraction kit.

commonly encountered when one uses conventional techniques. The protocol is rapid, stated to take less than 45 min, and provides DNA suitable for RAPD and ALFP analysis from a variety of plant species.

**Reader Enquiry No. 116**

### Recovery and isolation

CPG now offers a **total RNA isolation kit** designed to isolate pure, intact RNA from tissues and cells in less than 90 minutes. This kit utilizes guanidine thiocyanate and β-mercaptoethanol to effectively disrupt cellular structure while

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inactivating RNase released from membrane-bound organelles. Total RNA is isolated, free from protein and DNA, with a phenol:chloroform:isoamyl alcohol solution, a modification of a single-step protocol developed by Chomczynski and Sacchi. The kit contains enough reagents for approximately ten isolations, each isolation from one gram of tissue or  $1 \times 10^8$  cells. The purified RNA isolated by this kit is ready for use with downstream applications, such as mRNA isolation, northern blotting and RT-PCR.

**Reader Enquiry No. 117**

For fast recovery of DNA and RNA of any size, Epicentre has introduced the GELase agarose-gel-digesting preparation. This preparation can recover intact DNA and RNA in from LMP-agarose gels from amplification products of less than 100 bp to multi-megabase genomic DNA. Agarose digestion is said to be complete in as few as 8 minutes. Moreover, the manufacturer states that the simple procedure avoids organic extractions, shearing, low recovery and the introduction of contaminants that often occur using other methods. A GELase technical information brochure is available on request.

**Reader Enquiry No. 118**

## Labware

Specifically developed to meet the demands of hybridization applications, the SI 20H from Stuart Scientific is a hybridization oven with a 20-l capacity, incorporating both a rotisserie and rocking shaker. When used with 20-cm hybridization membranes, the oven can accommodate seven, 35-mm diameter bottles at a time (six for hybridization plus one for buffer solution). The rotisserie gently rotates at a speed that can be varied between 2 and 10 r.p.m. and can be easily lifted out, to serve as a bottle stand. With the rotisserie removed, a platform fitted in the back of the oven drops forward to form a rocking shaker with variable speed of 5–70 oscillations per minute. Full access to the rocker is ensured by an 'up and over' style door, which also offers the additional advantage of saving bench space. This unit is designed to be easy to use and is compact, with a stainless steel interior, forced air circulation, digital temperature control and safety thermostat.

**Reader Enquiry No. 119**

Grant Instruments has introduced a **microplate heater** to accommodate the most commonly used 96-well microplates and cell culture plates. Compact and flexible, the QBTM is designed to afford laboratory personnel the convenience of having a 'personal' heater for use with their favourite 96-well microplate. The QBTM offers pre-

cision temperature control for techniques requiring incubation or sample preparation within the range ambient  $\pm 5$  °C to 100 °C. The dry heating system is said to eliminate the risk of sample contamination and dilution. Direct contact between the microplate and an aluminium heating plate, together with the 'oven' effect created by the lid should ensure fast, efficient heating and excellent sample uniformity. Digital control allows the temperature to be set with high reproducibility, while  $\pm 0.1$  °C stability and  $\pm 0.5$  °C uniformity should ensure the repeatability required by stringent quality control procedures. The microplate support can be removed and aluminium blocks inserted for heating microcentrifuge tubes. Because the QBTM accommodates two blocks, microcentrifuge tubes of different sizes can be heated at the same time. An extraction tool allows safe, easy removal of blocks even when they are hot and loaded with tubes. Other safety features include a double-layer lid that should ensure that the outer case remains cool during high-temperature operation, a user-resettable overtemperature cutout, and a recessed panel protecting the controls from spillages.

**Reader Enquiry No. 120**

Hybaid now offers its new **sealing systems for reliable 96-well, oil-free thermal cycling**. These systems are designed to simplify oil-free, thermal cycling procedures. This follows the introduction of two new sealing systems for 96-well plates. The new self-adhesive TD tape is designed to eliminate the possibility of carryover. In formulating the adhesive and the tape itself, care has been taken to select only inert materials. As a result, they do not interfere with reagents used within cycling procedures, and this facilitates the generation of high product yields. The heat-resistant tape is said to be easy to remove at the end of heated-lid protocols, whereas the OmniSeal TD mat offers a cost-effective alternate to the disposable tape system. Made from silicone, the mat fits snugly into the wells of a 96-well array. Downward pressure from the universal heated lid fitted to Hybaid thermal cyclers should provide an efficient seal between the wells and the mat.

**Reader Enquiry No. 121**

GATC has introduced its automated **membrane developing system**, which is designed to analyse membrane-bound



**QBTM microplate heater from Grant Instruments.**

biomolecules automatically. Computer controlled protocols and automatic scanning eliminate all manual steps. The system works with all available membranes, Southern, northern, western and dot-blots. The membrane carrying the biomolecules is placed onto the outer surface of a transparent cylinder, which slowly rolls through a low-volume bath containing the reagents. A thin film of reagent is sufficient to produce a signal; therefore, membranes as large as 30 cm by 50 cm can be developed with a minimum of reagents (15 to 20 ml). The entire detection protocol is computer-controlled: reagents are automatically pipetted from the reagent storage rack into the incubation bath, they are automatically changed and the entire process is temperature-controlled. The actual detection is done with colorimetric or fluorescent reporter molecules (NTB/BCIP, ECL, LumiPhos, Attophos and the like). A built in visible/fluorescent scanner digitizes the results and stores the image on a one-gigabyte hard disk. For further processing, data may be transferred electronically through an integrated network port or by floppy disk.

**Reader Enquiry No. 122**

GRI now offers Robbins Scientific products including **hybridization incubators** that have tri-directional rotation capability that moves liquid from one end of the tube to the other as rotation occurs.

This rigorous action allows small volumes of highly concentrated DNA probe solutions to be used, yielding greater hybridization efficiency. In addition, up to ten membranes can be probed simultaneously, without the use of separating meshes, and without compromising background noise. For the busy laboratory, the model 400 provides high sample-processing rates with a capacity of ten  $35 \times 300$  mm tubes or twenty  $35 \times 150$  mm tubes using the standard tube rotator. For extra large runs a special rotator that holds six  $75 \times 300$  mm bottles is available. These bottles are said to be well suited for processing membranes from DNA sequencing and other large format gels. Three special rotators are available for laboratories running large numbers of small-format blots, that hold 80 50-ml tubes, 200 15-ml tubes or 400 1.5-ml tubes.

**Reader Enquiry No. 123**



**Robbins Scientific hybridization oven.**

*These notes are compiled by Brendan Horton from information provided by the manufacturers. For more details, fill in the reader service card bound inside the journal.*